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Studies on the Biosynthesis of Cholesterol

10. MEVALONIC KINASE AND PHOSPHOMEVALONIC KINASE FROM LIVER*

BY H. R. LEVY† AND G. POPJÁK
Medical Research Council, Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W. 12

(*Received* 6 *October* 1959)

After the discovery that mevalonic acid was a key intermediate in the biosynthesis of cholesterol (Tavormina, Gibbs & Huff, 1956), it was shown that liver preparations (Popják, Gosselin, Gore & Gould, 1958) as well as yeast extracts (Amdur, Rilling & Bloch, 1957; Lynen, 1959) required adenosine triphosphate, Mg^{2+} or Mn^{2+} and reduced pyridine nucleotides to convert mevalonate into squalene. The need for adenosine triphosphate suggested the formation of phosphorylated intermediates from mevalonate; the first ones to be identified were 5-phosphomevalonate and 5-diphosphomevalonate in yeast (Tchen, 1957; Chaykin, Law, Phillips, Tchen & Bloch, 1958; Lynen, 1959) as well as in rat-liver preparations (de Waard, 1958; de Waard & Popják, 1959; cf. Popják, 1959).

We have isolated from pig liver the enzyme catalysing the first of these phosphorylations and studied some of its properties. Shortly after this work was begun Tchen (1958) reported the partial purification of the same enzyme from yeast and named it 'mevalonic kinase'. During our purification procedures we encountered protein fractions which formed not only the 5-phosphomevalonate (5-P-MVA), but also the 5-diphosphomevalonate (mevalonic acid 5-pyrophosphate; 5-PP-MVA) from mevalonate and adenosine triphosphate. When 5-phosphomevalonate was added to such enzyme fractions, together with adenosine triphosphate and Mg^{2+}, 5-PP-MVA was obtained according to the reaction:

$$\text{5-P-MVA} + \text{ATP} \xrightarrow{Mg^{2+}} \text{5-PP-MVA} + \text{ADP}.$$

This second enzyme, which we propose to call 'phosphomevalonic kinase', was completely removed from mevalonic kinase. A brief account of this work has already been published (Levy & Popják, 1959). A large-scale preparation of 5-phospho[2-^{14}C]mevalonate with mevalonic kinase is now described also.

* Part 9: de Waard & Popják (1959).

† Postdoctoral Fellow of the National Cancer Institute, National Institutes of Health, United States Public Health Service.

MATERIALS AND METHODS

Biochemical reagents. The following compounds and enzymes were obtained from the Sigma Chemical Co., St Louis, Missouri, U.S.A.: adenosine triphosphate (ATP), cytidine triphosphate, guanosine triphosphate and inosine triphosphate; reduced diphosphopyridine nucleotide (DPNH), about 65% pure; sodium phosphoenolpyruvate (PEP); crystalline phosphoenolpyruvic kinase from muscle (PEP-kinase); lactic dehydrogenase from rabbit muscle and 2-amino-2-hydroxymethylpropane-1:3-diol (tris). Adenosine diphosphate (ADP) was the preparation of Pabst Laboratories, Milwaukee, Wisconsin, U.S.A. Protamine sulphate (salmine sulphate) was obtained from British Drug Houses Ltd., Poole, Dorset, and crystalline plasma albumin from Armour and Co., Chicago, U.S.A.

DL-*Mevalonic acid lactone and other substrates.* DL-Mevalonic acid lactone, synthesized according to the method of Cornforth, Cornforth, Popják & Gore (1958), was the gift of Dr J. W. Cornforth and Dr Rita H. Cornforth. It was converted into the potassium salt by dissolving in water, adding a small excess of KOH and keeping for 30 min. at 37°. DL-[2-^{14}C]Mevalonolactone was obtained from the Radiochemical Centre, Amersham, Bucks, and was also converted into the potassium salt; its specific activity was adjusted to 0·2 μc/μmole by dilution with unlabelled material. The concentration of the potassium DL-mevalonate (whether labelled or not) solutions was 0·1 M.

The specimens of the anhydro analogues of mevalonate [*cis*-5-hydroxy-3-methylpent-2-enoic acid and 5-hydroxy-3-methylpent-3-enoic acid (mixture of *cis* and *trans*)] were those used in previous work (Cornforth *et al.* 1958). 3-Hydroxy-3-methylglutaric acid, 3-oxobutan-1-ol and farnesoic (farnesinic) acid were the generous gifts of Dr J. W. Cornforth and Dr Rita H. Cornforth. The farnesoic acid (3:7:11-trimethyldodeca-2:6:10-trienoic acid, mixture of *trans–trans* and *cis–trans* acids) was prepared by the slow hydrolysis (at 4°) with KOH of ethyl farnesoate synthesized from *trans*-geranylacetone. Gas–liquid chromatography of the ethyl farnesoate showed the presence, in equal amounts, of two components only, corresponding to the esters of the *trans–trans* and *cis–trans* acids. 3:7:11-Trimethyldodecanoic acid was prepared by the catalytic hydrogenation of the ethyl farnesoate preparation, followed by saponification and extraction of the free acid; it was used as the potassium salt. Gas–liquid chromatography of the methyl ester of this acid showed a single component. A specimen of this same substance was also generously provided by Dr Karl Folkers of the Merck, Sharp and Dohme Research Laboratories, Rahway, New

Jersey, U.S.A. The method of preparation of this specimen is not known to us, but it was identical with our preparation and was of the same purity as determined by gas–liquid chromatography. A specimen of farnesol, prepared by the allylic rearrangement of nerolidol, was kindly provided by Dr M. Stoll of Firmenich et Cie, Geneva, Switzerland; it contained 56·8% of *trans–trans*-farnesol, 31·8% of *cis–trans*-farnesol, and 11·4% of nerolidol as revealed by gas–liquid chromatography.

Chromatographic procedures. The methods of chromatographic separation of mevalonic acid, mevalonic acid lactone, 5-phosphomevalonate and of 5-diphosphomevalonate on paper and on Dowex-2 formate ion-exchange resins were those previously described (de Waard, 1958; de Waard & Popják, 1959). The principal solvent system in paper chromatography used by us was the *iso*butyric acid–aq. NH_3 soln. (sp.gr. 0·88)–water system (66:3:30, by vol.). The paper-chromatographic strips were analysed for radioactive spots with an automatic scanning device as described previously (Hele, Popják & Lauryssens, 1957); the areas under the curves obtained in such records are proportional to the total radioactivity contained in the spots.

Assay of ^{14}C. The measurements of ^{14}C were made with thin mica-window Geiger counters on samples of 2 cm.^{2} area and negligible thickness. The efficiency of counting was about 6%.

Crude enzyme preparations. The dialysed soluble protein fraction of rat-liver homogenates [after precipitation with $(NH_4)_2SO_4$] described previously (Popják *et al.* 1958), was used in the early experiments, but purification of mevalonic kinase from such extracts was not attempted.

For the purification and large-scale preparation of mevalonic kinase, pig liver, obtained immediately after slaughter and brought to the laboratory on ice, was extracted according to the method of Finch & Hele (1959) with a sucrose medium of the following composition: sucrose, 0·35 M; $KHCO_3$, 0·035 M; ethylenediaminetetra-acetate, 0·001 M; and KCl, 0·025 M. In this method the liver, in 500 g. batches, is finely chopped with a sharp knife and is then extracted with 2 vol. of the sucrose medium by gentle stirring for 90 sec., and the large particles are removed from the extract by filtering through two layers of cheese-cloth. The volume of this filtrate ('the initial extract') was about the same as the volume of the sucrose medium used and contained 60–80 mg. of protein/ml. as determined by the biuret reaction (Gornall, Bardawill & David, 1949); it served as the starting material for the purification. Its further treatment is described under Results. All procedures of extraction and fractionation were done at 0–5°.

The calcium phosphate gel used for adsorption of protein was made according to the method of Tsuboi & Hudson (1957). All chemicals not specifically mentioned were of analytical grade.

Enzyme assays. Two methods were used. In the first method ('radiochemical assay') the amount of the phosphorylated derivative(s) formed from ^{14}C-labelled substrates (DL-[2-^{14}C]mevalonate, 5-phospho[2-^{14}C]mevalonate) was measured by paper chromatography of samples of the incubation mixtures deproteinized with $HClO_4$ as described previously (de Waard & Popják, 1959). Since mevalonic acid, mevalonic acid lactone, 5-phospho- and 5-diphospho-mevalonate are clearly separated from one another on the chromatograms, the percentage conversion of added ^{14}C-labelled substrate into new products could be readily obtained from the areas under the curves obtained by our scanning procedure with an accuracy of about ±5% (cf. under chromatographic procedures). When enzymic activity was measured by the radiochromatographic assay alone, the reactants contained in the incubation mixtures were the same and of the same final concentration as described for the spectrophotometric assay (see below) except that phosphoenolpyruvate, pyruvic kinase, lactic dehydrogenase and DPNH were omitted.

The spectrophotometric assay of liver mevalonic kinase was made by the same procedure as described by Tchen (1958) and by Lynen (1959) for the yeast enzyme. This assay measures the amount of ADP formed in the kinase reaction (1) by coupling with PEP-kinase (2) and lactic dehydrogenase reactions (3) as shown below.

$$\text{MVA} + \text{ATP} \rightarrow \text{5-P-MVA} + \text{ADP} \quad (1)$$
$$\text{ADP} + \text{PEP} \rightarrow \text{ATP} + \text{pyruvate} \quad (2)$$
$$\text{Pyruvate} + \text{DPNH} + \text{H}^+ \rightarrow \text{lactate} + \text{DPN}^+ \quad (3)$$
$$\text{Sum:} \quad \text{MVA} + \text{PEP} + \text{DPNH} + \text{H}^+ \rightarrow \text{5-P-MVA} + \text{lactate} + \text{DPN}^+$$

Each mole of mevalonate phosphorylated with ATP yields 1 mole of ADP (Tchen, 1958); this is rephosphorylated by phosphoenolpyruvate and PEP-kinase (reaction 2) and the pyruvate formed is used to oxidize DPNH in the presence of lactic dehydrogenase (reaction 3). The last reaction was followed at 340 mμ in a Beckman DK-2 recording spectrophotometer fitted with a time drive. The spectrophotometer cuvettes were held at 37° in a thermostatically-heated cell carrier constructed in the Unit's workshop (unpublished work).

The reaction mixture (5·9 ml.) for the assays was made up in a tube kept at 37° and contained the following reactants (μmoles/ml. shown in parentheses): cysteine hydrochloride (10); KOH, for the neutralization of cysteine hydrochloride (16); 2-amino-2-hydroxymethylpropane-1:3-diol–HCl (tris buffer), pH 7·4 (100); $MgCl_2$ (5); ATP (7); DPNH (0·2); sodium phosphoenolpyruvate (0·5). Lactic dehydrogenase and phosphoenolpyruvic kinase were combined and diluted with 1% (w/v) solution of bovine albumin; 0·2 ml. of this solution, containing 52 and 200 μg. of the two enzymes respectively, was used in the reaction mixture. The mevalonic kinase (0·1 ml.) was added last. After mixing, 2·9 ml. of the reaction mixture were transferred to the test cuvette and the remainder to the control cuvette. After recording the light-extinction at 340 mμ for 2 or 3 min. 0·1 ml. of mevalonate solution (usually 10 μmoles of the DL-substrate) was added to the test cuvette to initiate the reaction. The amount of DPNH used in the assay was less than the total amount of utilizable substrate; this circumstance in no way invalidated the assay since during the purification procedures we were interested only in rates of reaction in order to determine the specific activity of our preparations, there being no necessity to carry the reaction to completion. In order to ensure that the rate of DPNH oxidation was actually a measure of the rate of mevalonate phosphorylation, and

Table 1. *Purification of mevalonic kinase from extract of pig liver*

The enzymic activity was measured by the spectrophotometric assay.

Stage of purification	Amount of protein (mg.)	Enzyme units*	Specific activity of protein†
Initial extract	247 000	2180	0·0088
Supernatant after addition of protamine	75 000	2370	0·0315
Enzyme after heat treatment	37 000	1540	0·0415
After ammonium sulphate fractionation (30–45% saturation)	11 300	1200	0·106
Enzyme after calcium phosphate-gel treatment:			
1st eluate	1 021	668	0·654
2nd eluate	557	541	0·972

* μmoles of DPNH oxidized (= μmoles of mevalonate phosphorylated)/min. at 37°.
† μmoles of DPNH oxidized/min./mg. of protein at 37°.

was not limited by the lactic dehydrogenase and pyruvic kinase-catalysed reactions, the system was tested when necessary by the addition of about 1 μmole of ADP to the test cuvette; this caused an almost instantaneous oxidation of DPNH. It was particularly important to test the assay system in this way in those experiments in which the properties of mevalonic kinase were being studied since some of the conditions used could, conceivably, have affected the test reactions. Whenever the spectrophotometric assay could not be used, e.g. during the study of metal-ion and nucleotide requirements of mevalonic kinase, the 'radiochromatographic assay' was employed.

RESULTS

As the formation of 5-phosphomevalonate with liver enzymes was demonstrated in our Laboratory first in extracts of rat liver (de Waard, 1958, de Waard & Popják, 1959), supernatants of rat-liver homogenates centrifuged at 105 000 *g* were first used as possible sources of mevalonic kinase. Although it was possible to demonstrate spectrophotometrically the presence of mevalonic kinase in such preparations, on account of rapid utilization of 5-phosphomevalonate in further reactions (cf. de Waard & Popják, 1959; Popják, 1959) and because of other interfering reactions in the rat-liver extracts the assay was difficult. In the course of experiments on the preparation of amino acid-activating enzymes (Finch & Hele, 1959) Dr P. Hele of this Unit noted the presence of mevalonic kinase in extracts of pig liver; the enzyme could be assayed spectrophotometrically even in the crudest of extracts (the 'initial extract' described under Methods). As these assays could be readily reproduced and the 'radiochromatographic assay' also (see under Methods) showed an excellent conversion of [2-^{14}C]mevalonate into 5-phospho[2-^{14}C]mevalonate, extracts of pig liver were used for purification.

Purification of mevalonic kinase

Table 1 shows the results of purification of mevalonic kinase from 3·46 l. of pig-liver extract. The 'initial extract' was treated in 1–1·2 l. batches with a solution of protamine sulphate (10 mg./ml.) to give a final concentration of 1·5 mg. of protamine/ml. of extract. This concentration of protamine was found in separate small-scale experiments to lead to a maximum increase in specific activity without loss of enzyme. The mixture was stirred slowly for 2 min. and then centrifuged to give the almost clear 'protamine supernatant' (3·16 l.). As is shown in Table 1 the protamine treatment resulted in a 3·5-fold increase in the specific activity of the preparation.

Solid ammonium sulphate (1·76 kg.) was added to 3·16 l. of 'protamine supernatant' to give a concentration of 85% saturation at 0°; the precipitate was allowed to settle overnight and collected by centrifuging. The reddish brown precipitate was dissolved in 1365 ml. of a 0·02 M-potassium hydrogen phosphate buffer, pH 7·6, containing mM-ethylenediaminetetra-acetate (EDTA); a small insoluble residue was centrifuged off.

At this stage of purification several small samples of the preparation were heated to various temperatures (up to 60°) in the absence or presence of mM-mevalonate. The results encouraged us to heat the bulk of the enzyme with added mevalonate (1 mM), in 200 ml. lots, at 55° for 2 min.; the preparation was cooled immediately afterwards in ice. A large precipitate was centrifuged off and washed with the dilute phosphate–EDTA buffer, and the washings were added to the supernatant. In contrast with the good recovery and an increased specific activity of the enzyme in small-scale experiments, heating the enzyme in bulk caused relatively large losses with only a little gain in specific activity (cf. Table 1; enzyme after heat treatment).

After heat treatment the protein solution (1850 ml.) was subjected to ammonium sulphate fractionation; about 80% of the enzyme precipitated between 30 and 45% ammonium sulphate saturation with an over twofold increase in specific activity. The precipitate, containing the mevalonic

kinase, was dissolved in 288 ml. of the dilute phosphate–EDTA buffer and was dialysed for 3½ hr. against two changes of 0·01 M-potassium hydrogen phosphate buffer, pH 7·5. The dialysed protein solution (335 ml.) was stirred with 35 ml. of a suspension of calcium phosphate gel (35 mg. dry weight/ml. of suspension) for a few minutes; the gel was centrifuged off and the supernatant solution assayed for protein concentration and enzyme activity. This first gel treatment removed much inert protein from solution and no enzyme. The supernatant from the first gel treatment was stirred with further portions of gel, which began to adsorb the enzyme; after the addition of 175 ml. of gel suspension 95% of the mevalonic kinase was adsorbed; the supernatant after centrifuging was almost completely inactive and was discarded.

The calcium phosphate gel, containing the adsorbed mevalonic kinase, was washed successively with approximately equal volumes of potassium hydrogen phosphate buffers, pH 7·5, and once with 0·01 M- and twice with 0·02 M-buffer. These washings removed inert protein only. Four washes with 0·05 M-potassium hydrogen phosphate buffer, pH 7·5, eluted mevalonic kinase quantitatively from the gel; these washes were combined in two fractions to give the '1st eluate' and the '2nd eluate' shown in Table 1. The enzyme was precipitated with ammonium sulphate (80% saturation), the precipitate collected by centrifuging and the supernatant decanted. The precipitated enzyme was stored at −15°. When needed, solutions were made in 0·02 M-phosphate buffer, pH 7·5, and dialysed before use against the same buffer for 4 hr. Solutions of the enzyme may be kept at −15° for several weeks with relatively little deterioration.

The over-all yield of the two calcium phosphate gel eluates was 56%, based on the enzyme units contained in the initial extract; the '1st eluate' contained mevalonic kinase purified 74-fold and the '2nd eluate' 110-fold. In another preparation (nos. 8–29), similar to this one, the final product phosphorylated 1·2 μmoles of (+)-mevalonate/min./mg. of protein at 37°; that preparation was used for the study of the properties of the enzyme which follow.

Properties of mevalonic kinase

The final spectrophotometric assay system described under Methods was arrived at after the properties of mevalonic kinase in the most highly purified preparation (specific activity 1·2 μmoles of mevalonate phosphorylated/min./mg. of protein at 37°) were studied. The accuracy of this assay on 'initial extracts' is probably not very great. We have observed during the use of this assay on crude enzyme preparations a fairly rapid 'back-reaction' after the DPNH has been partially oxidized. The product of this reaction had an absorption maximum at 340 mμ and was presumably DPNH. This back-reaction did not depend on mevalonic acid; it also occurred when ADP was used as substrate. This phenomenon, which disappeared during further purification of the enzyme, may account for the apparent rise in total enzyme units after protamine treatment (cf. Table 1). After this step of purification, however, the assays became reproducible and the rates of reaction proportional to protein concentration. This is shown in Fig. 1*b* for a partially purified preparation with a specific activity of about 0·15 μmole/min./mg. of protein; in Fig. 1*a* the actual spectrophotometric records, from which the points on Fig. 1*b* were calculated, are reproduced. Similar data were obtained also with the more highly purified preparations.

Activation of mevalonic kinase by cysteine. The effect of cysteine on the activity of the enzyme is shown in Fig. 2. In the absence of cysteine the rate of reaction was only 17% of the rate found in the presence of 0·66 μmole of cysteine/ml., the lowest concentration tested. A 20-fold increase in cysteine concentration resulted in a further 25–30% rise in activity. Glutathione was almost as effective as cysteine, both at a concentration of 10 μmoles/ml.,

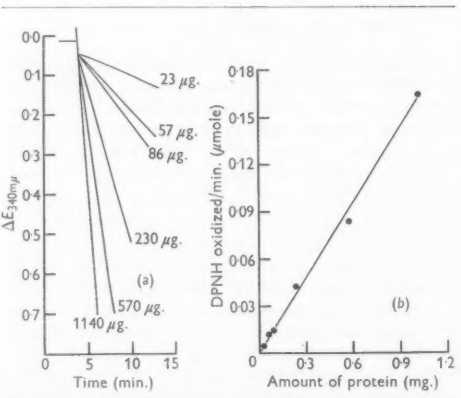

Fig. 1. Proportionality between protein concentration and phosphorylation of mevalonate as measured spectrophotometrically by the phosphoenolpyruvic kinase–lactic dehydrogenase-coupled assay system. In (*a*) are shown copies of the spectrophotometric records from which the data of (*b*) were calculated. The spectrophotometric assays were set up exactly as described under Methods, the amounts of enzyme protein (preparation no. 5–36) being varied from 23 to 1140 μg. per assay as indicated on graph (*a*). The upward stroke after the initial recording of light-extinction indicates the addition of mevalonate to the test cuvettes. The pen of the recording spectrophotometer was brought to the same starting point for each assay and the mevalonate was added also at the same point in each test.

but ascorbic acid was without effect. Cysteine or glutathione was needed, however, only when air was not excluded from the incubations.

Addition of mM-*p*-chloromercuribenzoate produced an 80% inhibition of mevalonic kinase in the presence of mM-cysteine as measured by the radiochromatographic assay. This inhibition was completely reversed by raising the concentration of cysteine to 0·02M. Iodoacetamide did not inhibit the enzyme at either mM or 10 mM concentration.

Metal-ion requirements of mevalonic kinase. The enzyme needs a bivalent metal ion for activation, as was shown by both spectrophotometric and radiochromatographic assays. Fig. 3 shows the effect of varying Mg^{2+} and Mn^{2+} ion concentrations on enzyme activity as determined by the spectrophotometric assay. The validity of these assays was established by the fact that even at the lowest concentrations of Mn^{2+} and Mg^{2+} ion used the pyruvic kinase–lactic dehydrogenase assay system was fully responsive, i.e. addition of ADP caused an almost instantaneous oxidation of DPNH. A correlation was established between the concentration of Mg^{2+} ion and ATP and enzyme activity (see under Nucleotide requirements). The data of Fig. 3 were obtained with an ATP concentration of 4·2 μmoles/ml.; when the ATP concentration was raised the curve showing enzyme activity against Mg^{2+} concentration was shifted upward and to the right.

Besides Mg^{2+} and Mn^{2+} other bivalent metal ions, Ca^{2+} (as the chloride), Ba^{2+}, Zn^{2+} (as the acetates), Co^{2+} (as the nitrate), Fe^{2+} and Cu^{2+} (as the sulphates), were also tested by the radiochromatographic assay for their ability to activate mevalonic kinase. In a representative experiment eight 1 ml. incubations, each containing one of the above metal ions in a concentration of 5 mM (5 μmoles/ml.), were set up with 1 μmole of DL-[2-^{14}C]mevalonate, 7·7 μmoles of ATP, 10 μmoles of cysteine (neutralized with potassium hydroxide), 100 μmoles of tris–HCl buffer, pH 7·4, and 62 μg. of purified mevalonic kinase. After 5 min. of incubation at 37° the contents of each incubation were deproteinized and samples of each chromatographed on Whatman no. 1 paper. The records obtained from scanning the paper chromatograms for radioactivity showed that with Mg^{2+} 0·368, with Mn^{2+} 0·116 and with Ca^{2+} 0·214 μmole of 5-phosphomevalonate was formed; a trace of 5-phosphomevalonate formed in the presence of Fe^{2+} and Co^{2+}, and none in the presence of Ba^{2+}, Cu^{2+} and Zn^{2+} ions. The activation of mevalonic kinase by Ca^{2+} ions was checked and confirmed in further experiments. The calcium chloride solution used was analysed in a flame spectrophotometer and was found to be essentially free of Mg^{2+} ions. Ca^{2+} in a concentration of 5 mM did not inhibit mevalonic kinase in the presence of 5 mM-Mg^{2+} ions.

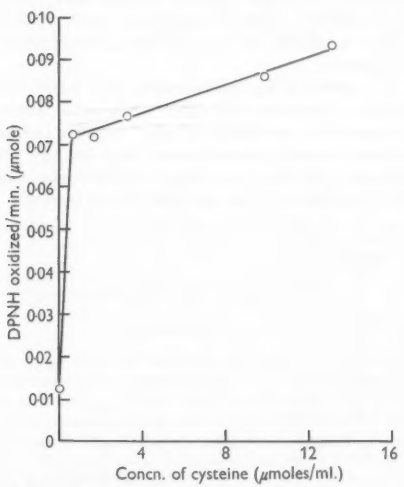

Fig. 2. Effect of cysteine on mevalonic-kinase activity as measured by spectrophotometric assay. The spectrophotometric assays for each point on the graph were set up as described under Methods, except that the concentration of cysteine was varied from 0–13 μmoles/ml. Enzyme preparation no. 8–29 was used (93 μg. of protein per assay).

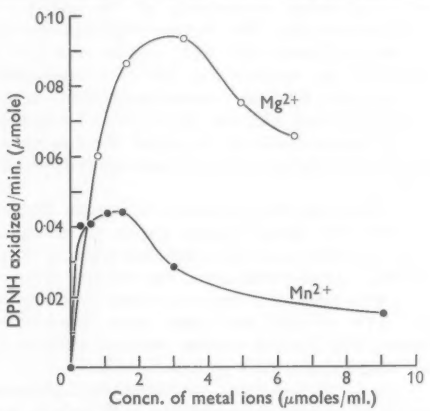

Fig. 3. Effect of Mg^{2+} and Mn^{2+} ions on mevalonic-kinase activity as determined spectrophotometrically. The spectrophotometric assays for each point on the graphs were set up as described under Methods, except that the concentration of ATP was 4·2 μmoles/ml., instead of the usual 7·5 μmoles/ml., and the concentration of Mg^{2+} and Mn^{2+} ions varied from 0 to 9 μmoles/ml. Enzyme preparation no. 8–29 was used (93 μg. of protein per assay).

Nucleotide requirements of mevalonic kinase. The effects of varying concentrations of ATP on mevalonic-kinase activity was first measured by the spectrophotometric assay. The optimum concentration of ATP depended on the concentration of Mg^{2+} ions in the incubations. Thus with 3·3 mM-Mg^{2+} the apparent optimum concentration of ATP was 5·5 mM, and with 5 mM-Mg^{2+} the optimum concentration of ATP was 7–8 mM. With an ATP concentration higher than 8 mM the coupled spectrophotometric assay could not be used because of an almost complete inhibition of pyruvic kinase. Mevalonic kinase, on the other hand, was not inhibited by higher concentrations of ATP as judged by the results of the radiochromatographic assays. The optimum ratio of ATP to Mg^{2+} (μmole/μmole) for maximum rates of mevalonic-kinase activity was found by the spectrophotometric assay to be 1·5–2.

A number of nucleotides, adenosine diphosphate, cytidine triphosphate, guanosine triphosphate and inosine triphosphate were tested, in a concentration of 7·4 mM in the presence of 5 mM-Mg^{2+}, for their ability to replace ATP in the phosphorylation of mevalonate. The experiments were set up essentially in the same way as for the tests with the various metal ions, except that the incubations lasted for 15 min. The radiochromatographic assay showed that only inosine triphosphate could replace ATP; the amount of 5-phosphomevalonate formed with inosine triphosphate was very nearly identical with that formed in the presence of ATP, 41 and 50% conversion respectively of the added DL-[2-^{14}C]mevalonate. The inosine triphosphate was not contaminated with ATP, neither was it converted by the enzyme into ATP, for paper chromatograms of the deproteinized incubation mixture revealed neither ATP nor ADP. These nucleotides are well recognizable on the paper chromatograms developed with the *iso*butyric acid–aq. NH_3 solvent system.

In crude enzyme preparations sodium fluoride (0·012 M) was added during assays of mevalonic kinase in order to inhibit adenosine triphosphatase activity. After purification, the enzyme preparations were tested for enzymes which could hydrolyse ATP to ADP and these were found to be absent. The purified enzyme was not affected by the addition of 0·012 M-sodium fluoride.

Effects of substrate concentrations; substrate specificity. When DL-mevalonic acid lactone was used as substrate with the most highly purified mevalonic kinase the reaction rate, as measured spectrophotometrically, was at first very slow, but it gradually increased. If, instead of lactone, the potassium salt of mevalonic acid was used as substrate, a rapid reaction set in immediately after its addition to the incubation mixture without any subsequent change in rate. Clearly the enzyme cannot utilize mevalonic acid lactone; the slow reaction rates observed when the lactone was used as substrate obviously depended on the rate of hydrolysis of the lactone to the acid at the slightly alkaline pH of the incubations.

The effect of the concentration of substrate (potassium DL-mevalonate) on the rate of its phosphorylation is shown in Fig. 4; these data were derived from spectrophotometric assays. A maximum rate was observed when the concentration of DL-mevalonate was about 2 μmoles/ml. As is shown below (cf. Fig. 5), liver mevalonic kinase, like the yeast enzyme (Tchen, 1958), acts on only one of the optical enantiomorphs of mevalonate. If we assume that the binding of the active enantiomorph by the enzyme was not affected by the presence of the unnatural enantiomorph, the apparent Michaelis constant (K_m) for the natural isomer is approximately 50 μM.

In another experiment the spectrophotometric assay was set up in such a way that the amounts of phosphoenolpyruvate (1·5 μmoles) and of DPNH (1·3 μmoles) were in excess of the amount of DL-mevalonate (0·4 and 0·1 μmole) added to the reaction mixture. As can be seen from Fig. 5, after the addition of 0·4 μmole of DL-mevalonate the reaction came to a standstill when 0·21 μmole of DPNH was oxidized; after the addition of a further 0·1 μmole of DL-mevalonate 0·05 μmole of DPNH was oxidized. Thus it appears that mevalonic kinase is specific for one optical enantiomorph of mevalonate.

In addition to mevalonate we have tested related substances whether they can serve as substrate for mevalonic kinase. Neither the *cis*-5-hydroxy-3-methylpent-2-enoic acid (as the potassium salt), nor the 5-hydroxy-3-methylpent-3-enoic acid (mixture of the *cis* and *trans* isomers) (cf.

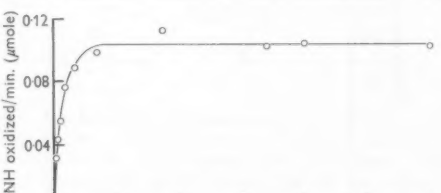

Fig. 4. Effects of substrate concentration on phosphorylation of mevalonate as measured by the spectrophotometric assay. The assays for each point on the graph were set up as described under Methods, except that the concentration of DL-mevalonate was varied from 0·035 to 6 μmoles/ml. Enzyme preparation no. 8–29 (93 μg. of protein per assay) was used.

Cornforth *et al.* 1958), nor 3-oxobutan-1-ol nor farnesol could be phosphorylated by mevalonic kinase; none of these substances inhibited the phosphorylation of mevalonate.

pH *optimum for mevalonic kinase.* Fig. 6 shows the dependence of rate of reaction on pH in phosphate and tris–HCl buffers as determined by the spectrophotometric assay. The pH optimum lies close to 7·3; at pH 6 and 8·7 the rate of phosphorylation of mevalonate was approximately one-half of that found at pH 7·3. In the pH range studied, 6–9·1, the rates of the pyruvic kinase–lactic dehydrogenase assay system did not limit the assay of ADP formed in the mevalonic kinase reaction.

Action of compounds related to mevalonic acid and to farnesoic acid on mevalonic kinase. In addition to the two anhydro-analogues of mevalonate, 3-oxobutan-1-ol and farnesol, we have tested the following substances (in a final concentration of 1 mM) for their effect on mevalonic kinase: 3-hydroxy-3-methylglutarate, *iso*valerate, farnesoate (mixture of the *cis–trans* and *trans–trans* acids) and 3:7:11-trimethyldodecanoate (each as the potassium salt). Of these only the farnesoate and the 3:7:11-trimethyldodecanoate had an effect on mevalonic kinase: the former caused a 25% and the latter a 95% inhibition of the enzyme. These two acids were without effect on the pyruvic kinase–lactic dehydrogenase system.

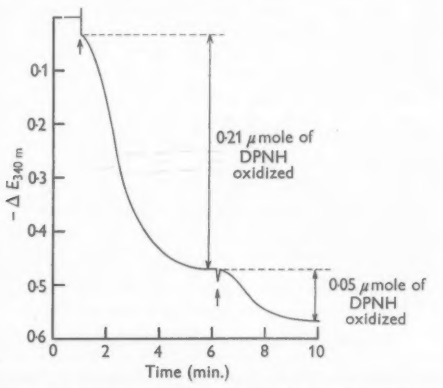

Fig. 5. Experiment illustrating specificity of mevalonic kinase for one optical enantiomorph of DL-mevalonate. The graph is a copy of the spectrophotometric recording. Spectrophotometric assay was set up with enzyme preparation no. 8–29 A (specific activity 0·5 μmole/min./mg. of protein). The test cuvette contained (in 3 ml.) 0·58 mg. of protein and the usual reactants of the spectrophotometric assay (see under Methods) except that 1·2 μmoles of phosphoenolpyruvate and 1·2 μmoles of DPNH were used in the assay. At the first arrow (at 1 min.) 0·4 μmole and at the second arrow (after 6 min.) 0·10 μmole of DL-mevalonate was added. The slight lag in the starting of the reaction after the two additions of mevalonate is due to the sub-optimum concentration of substrate.

5-*Phosphomevalonic kinase*

In addition to 5-phosphomevalonate the formation of another phosphorylated derivative of mevalonic acid was observed both in yeast extract (Chaykin *et al.* 1958) and in rat-liver extracts (de Waard, 1958; Popják, 1959; de Waard & Popják, 1959). Chaykin *et al.* (1958) showed by the use of [^{32}P]ATP and de Waard & Popják (1959) by the direct chemical analysis of the substance isolated that it contained 2 atoms of P/molecule of mevalonic acid. Although this substance still requires further chemical characterization, Chaykin *et al.* (1958) deduced from data of isotopic labelling with [^{32}P]ATP that it is most probably the 5-pyrophosphoryl derivative of mevalonic acid. We refer below to this substance as the 5-diphosphomevalonate. 5-Diphosphomevalonate is clearly separated from 5-phosphomevalonate both on paper chromatograms with the *iso*butyric acid–aq. ammonia solvent system and on Dowex-2 or Amberlite IRA-400 ion-exchange resins (cf. Popják, 1959; de Waard & Popják, 1959).

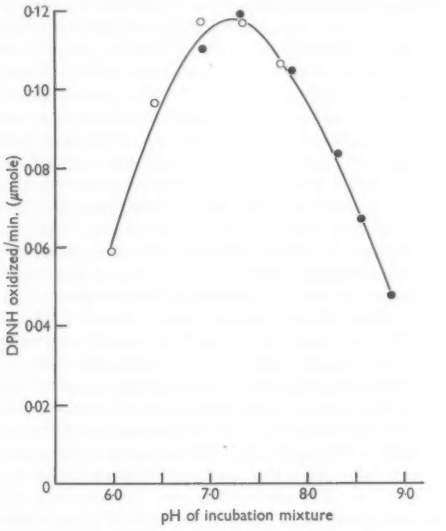

Fig. 6. Dependence of mevalonic-kinase activity on pH of reaction mixture as determined in spectrophotometric assays. The assays for each point have been set up as described under Methods. ○, Phosphate buffers, 0·1 M; ●, tris–HCl buffers, 0·1 M. Enzyme preparation no. 8–29 was used.

We have observed in the radiochromatographic assays of some of the cruder mevalonic-kinase preparations that in addition to 5-phosphomevalonate some 5-diphosphomevalonate was also formed. In the preparations obtained after calcium-gel fractionation 5-diphosphomevalonate was never formed. We have therefore assumed the existence of a second phosphorylating enzyme and in the following brief account we wish to present experimental evidence on this point, although this second enzyme has not been obtained free from mevalonic kinase.

A partially purified mevalonic-kinase preparation [no. 10–8; specific activity 0·22 μmole of (+)-mevalonate phosphorylated/min./mg. of protein] obtained after protamine treatment and ammonium sulphate fractionation of the 'initial extract' of pig liver, when incubated with [2-^{14}C]mevalonate and the usual components of the mevalonic-kinase assay system, was found to catalyse the formation not only of 5-phosphomevalonate but also of 5-diphosphomevalonate as determined by the radiochromatographic assay. A large-scale incubation (final volume 50 ml.) was set up with this enzyme preparation by using 75·6 mg. of protein, 100 μ-moles of DL-[2-^{14}C]mevalonate, 370 μmoles of ATP, 250 μmoles of $MgCl_2$, 5 m-moles of tris–HCl buffer, pH 7·4, 500 μmoles of cysteine (neutralized with potassium hydroxide), 23 μmoles of phosphoenolpyruvate and 1 mg. of phosphoenolypyruvic kinase. After 33 min. of incubation at 37° the mixture was deproteinized with an equal volume of ice-cold 10 % (w/v) perchloric acid solution and the acid removed as the insoluble potassium perchlorate. Radiochromatographic assays on 0·1 ml. samples of the deproteinized mixture showed that 49 % of the added DL-mevalonate was converted into 5-phosphomevalonate + 5-diphosphomevalonate. Of the phosphorylated derivatives 70 % was accounted for as 5-phosphomevalonate and 30 % as 5-diphosphomevalonate. The bulk of the extract was used for the purification of the two phosphorylated derivatives according to de Waard & Popják (1959) by using their method of ion-exchange chromatography followed by adsorption on and elution from charcoal. Two radiochemically pure specimens, uncontaminated by nucleotides or inorganic phosphorus, were obtained, the chromatographic properties and phosphorus contents of which were identical with those described by de Waard & Popják (1959) for 5-phospho[2-^{14}C]-mevalonate and 5-diphospho[2-^{14}C]mevalonate. These were used as substrates in further experiments.

Addition to enzyme preparation no. 10–8 of 5-phospho[2-^{14}C]mevalonate, together with ATP and Mg^{2+} ions, resulted in the formation of 5-diphospho[2-^{14}C]mevalonate. Both the spectrophotometric assay, as used for mevalonic kinase, and the radiochromatographic assay were satisfactory for measuring the activity of the second phosphorylating enzyme. Since the substrate for this enzyme is 5-phosphomevalonate we propose to name it '5-phosphomevalonic kinase' (or phosphomevalonic kinase).

In a spectrophotometric assay set up with 5-phospho[2-^{14}C]mevalonate as substrate (0·81 μmole/3 ml.) and 1 mg. of enzyme preparation no. 10–8, DPNH was oxidized at an initial rate of 0·018 μ-mole/min., but this rate fell off gradually. After 25 min. at 37° radiochromatographic assay was carried out on the deproteinized incubation mixture; this showed that 30 % (0·243 μmole) of the added 5-phospho[2-^{14}C]mevalonate was converted into the diphosphate. No hydrolysis of the 5-phospho[2-^{14}C]mevalonate into [2-^{14}C]mevalonate could be observed. Photographic prints of the paper chromatograms, made with ultraviolet light, revealed only ADP and ATP, but no adenosine monophosphate. Therefore we conclude that the reaction catalysed by phosphomevalonic kinase may be represented by:

$$\text{5-P-MVA} + \text{ATP} \xrightarrow{Mg^{2+}} \text{5-PP-MVA} + \text{ADP}$$

In the absence of Mg^{2+} ions 5-diphosphomevalonate was not formed from 5-phosphomevalonate; Ca^{2+} could not replace Mg^{2+} in the reaction, but neither did Ca^{2+} antagonize Mg^{2+}. In an experiment run parallel with the above spectrophotometric assay, in the presence of 5 mM-Ca^{2+} plus 5 mM-Mg^{2+}, 32 % (0·26 μmole) of the added 5-phospho[2-^{14}C]mevalonate was converted into 5-diphospho[2-^{14}C]mevalonate. In a further experiment we examined the possibility that the phosphomevalonic kinase reaction might be reversible; however, we could not demonstrate the formation of 5-phosphomevalonate and of ATP from 5-diphosphomevalonate and ADP in the presence of Mg^{2+} ions.

Preparation of 5-phospho[2-^{14}C]mevalonate

Although the method of de Waard & Popják (1959) is satisfactory for the preparation of the two phosphorylated derivatives of mevalonic acid in a pure form, the yields are rather poor on account of losses encountered during absorption on and elution from charcoal. We have developed a simple procedure, not involving chromatography, which is suitable for the large-scale preparation of 5-phosphomevalonic acid made enzymically from DL-mevalonate.

The method depends on the properties of 5-phosphomevalonic acid and of mevalonic acid. These are: (i) 5-phosphomevalonic acid is readily soluble in 85 % ethanol, but it forms a barium salt

sparingly soluble in water and insoluble in 80–85% ethanol; (ii) although mevalonic acid also forms a barium salt insoluble in ethanol, mevalonic acid lactone does not; (iii) free mevalonic acid may be converted into the lactone under dehydrating conditions and the lactone is fairly stable even at slightly alkaline pH at low temperatures. These characteristics of 5-phosphomevalonic acid and of mevalonic acid enable one to separate these two substances (*a*) from proteins, nucleotides and salts present in the enzymic incubations and (*b*) from one another.

In a large-scale experiment (final volume 500 ml.) we have used 40 mg. of our most highly purified enzyme preparation (no. 8–29, specific activity 1·2 μmoles of mevalonate phosphorylated/min./mg. of protein at 37°) and 2700 μmoles of DL-[2-^{14}C]-mevalonate (specific activity 0·2 μC/μmole) for the preparation of 5-phospho[2-^{14}C]mevalonate. The final concentrations of the reactants were: ATP, 7·5 mM; $MgCl_2$, 5 mM; cysteine (neutralized with potassium hydroxide), 1·8 mM; tris–HCl buffer, pH 7·3, 100 mM; DL-[2-^{14}C]mevalonate, 5·4 mM; enzyme 80 μg./ml. The reaction mixture, without mevalonate, was warmed to 37° and then the mevalonate added; the incubation was continued (without shaking) for 117 min. Radiochromatographic assay carried out at this time on a small sample of the incubation mixture showed that 42·5% of the DL-mevalonate [or 85% of the natural (+)-isomer] was converted into 5-phospho[2-^{14}C]-mevalonate. There was no trace of the 5-diphosphomevalonate.

The whole reaction mixture was frozen and dried from the frozen state. The dry residue was taken up in water, the pH adjusted to 4·0 with HCl and the volume made up to 45 ml. with water; ethanol (255 ml.) was then added to give a concentration of 85%. A large white precipitate was allowed to settle overnight and was then removed by centrifuging. The precipitate was washed repeatedly with 85% ethanol and the washings were added to the first ethanol supernatant (total volume 440 ml.). The ethanol was first removed from this solution on a rotary evaporator under vacuum at about 50–60°, then the aqueous solution was concentrated to a small volume by sublimation from the frozen state. The aqueous solution (41·5 ml.) was adjusted with potassium hydroxide to about pH 8·5 (phenolphthalein to pale pink), then 10 ml. of M-$BaCl_2$ solution and ethanol to a final concentration of 85% were added. A bulky white precipitate formed, which was allowed to accumulate at 0° overnight and was separated by centrifuging; the supernatant was drawn off and the precipitate washed with 85% ethanol. The washing was combined with the previous supernatant. The precipitate was dissolved in 25 ml. of N-HCl and the solution diluted with 25 ml. of water to give a volume of 53 ml. Radiochromatographic assay showed that the ethanol supernatant contained only unused mevalonic acid. Similar assays and ^{14}C-counting made on the solution of the barium salts revealed that about 91% of the ^{14}C counts in this solution were associated with 5-phosphomevalonate and 9% with mevalonate and that the 5-phosphomevalonate formed in the incubation was recovered quantitatively in this specimen. The chromatograms also showed that only a trace of ATP was present in the solution of the barium salts, most of the nucleotides having been left behind after the first ethanol extraction.

In order to remove the small amount of nucleotide left with the 5-phosphomevalonate, the solution of the barium salts (after removal of barium with sodium sulphate) was dried from the frozen state and the dry residue was extracted with 85% ethanol as in the first step and the barium precipitation repeated once again. Although all traces of nucleotide disappeared after this treatment, there still remained about 5% of mevalonate contaminating the 5-phosphomevalonate. The recovery of 5-phosphomevalonate was quantitative even after this second barium precipitation.

We now realized that in the further handling of the specimen we made mistakes, as at this stage of the work we did not appreciate fully the basis of the remarkably good separation of 5-phosphomevalonate from mevalonate in the first barium-precipitation step, i.e. the formation of a fairly stable lactone of free mevalonic acid during freeze-drying of a slightly acid solution. In the light of further experience, however, we now recommend that after the second barium precipitation the barium salts should be dissolved in dilute hydrochloric acid and the barium removed with sodium sulphate, followed by drying the specimen from the frozen state *in vacuo*. After this dried specimen, containing mostly free 5-phosphomevalonic acid and a little mevalonic acid, is heated *in vacuo* to about 60° the mevalonic acid lactonizes; the mixture is then dissolved in ice-cold water and the solution adjusted to pH 8·5 with a saturated solution of barium hydroxide while the specimen is kept packed in ice. Immediate addition of ethanol to 85% concentration then completes the precipitation of the barium salt of 5-phosphomevalonate, leaving mevalonic acid lactone in solution.

In order that others may avoid our mistakes after the second barium precipitation, we describe briefly the various procedures that we found ineffective in removing the small amounts of mevalonic acid that remained with the 5-phosphomevalonate. Extraction of an acid solution with ether did not remove mevalonic acid without some 5-phosphomevalonate as well. An attempt to sublime off the mevalonic acid from the freeze-dried specimen failed also;

by this time only 87% of the ^{14}C-counts contained in the specimen after the first barium precipitate were left, the 5-phosphomevalonate still being contaminated with about 5% of mevalonic acid. This specimen was chromatographed on Dowex-2 (formate) as described previously (de Waard & Popják, 1959) by using gradient elution with formic acid and ammonium formate. After collection of the 5-phosphomevalonate peak the fractions were combined and acidified with hydrochloric acid and most of the formic acid removed by extensive extraction with ether, the ammonium chloride being left behind. The aqueous solution was now made slightly alkaline and barium chloride added once more, followed by ethanol to 85%. The precipitate formed was collected by centrifuging and then treated in the usual way to remove the barium; the solution was then dried again from the frozen state. The dry residue was dissolved in a minimum of water, ethanol added to 85% and a large precipitate (ammonium chloride mostly) removed. Radiochromatographic analysis showed that the specimen now contained not even a trace of mevalonic acid, but only 750 μmoles of 5-phosphomevalonate out of the 1147 formed originally were recovered. After removal of the ethanol by evaporation the solution of 5-phosphomevalonic acid was kept at $-15°$, where it showed no signs of deterioration after some months. Phosphorus analyses on the specimens carried out according to a modification of the method of Allen (1940) showed that it contained 1 μg.atom of organic phosphorus per 31 500 counts/min. of ^{14}C; 1 μmole of the mevalonate used in the enzyme incubation contained 32 000 counts/min. The specimen contained no inorganic phosphorus. The losses encountered during these last procedures were due partly to the large number of manipulations and possibly to some hydrolysis of 5-phosphomevalonate to mevalonate; all this can now be avoided. The 5-phospho[2-^{14}C]mevalonate thus prepared was tested in enzymic reactions leading to the formation of further intermediates (notably of farnesyl pyrophosphate) in squalene biosynthesis and was utilized almost quantitatively in such reactions.

DISCUSSION

Extracts of pig liver made according to the method of Finch & Hele (1959) are much richer sources of mevalonic kinase than yeast extracts used by Tchen (1958); furthermore, even in the crudest pig-liver extracts, the 5-phosphomevalonate formed from mevalonate and ATP is metabolized only to the 5-diphosphomevalonate stage. This is in contrast with the soluble supernatants of rat-liver homogenates, in which after an incubation lasting longer than about 10 min. most of the 5-phosphomevalonate and 5-diphosphomevalonate formed during the first 5 min. may be completely dissipated into other products (cf. de Waard & Popják, 1959; Popják, Horning, Bucher & Cornforth, 1959). It seems probable that the advantages of the pig-liver extract derive from the gentle method of extraction, which does not release too many interfering enzymes. Possibly this initial advantage is reflected also in gaining preparations of mevalonic kinase of much higher specific activity than those reported by Tchen (1958) for yeast mevalonic kinase. The best preparation from yeast catalysed the phosphorylation of about 0·08 μmole of mevalonate/min./mg. of protein at 30°, as compared with 1·2 μmoles/min./mg. of protein at 37° for our best preparation. The recovery of mevalonic kinase from the yeast extracts was only about 7%; from the pig-liver extracts it was 56%.

Although yeast mevalonic kinase and liver mevalonic kinase are broadly similar, their properties differ in detail. Both enzymes appear to be SH-enzymes, but the liver enzyme is readily inactivated by molecular oxygen and needs cysteine or glutathione for reactivation under aerobic conditions; the yeast enzyme, according to Tchen (1958), was not affected by air or oxygen and did not need cysteine for activation. Although the complete squalene- and sterol-synthesizing system of rat liver (Popják *et al.* 1958) could be activated by cysteine, glutathione or ascorbic acid, the last substance was totally ineffective with mevalonic kinase; this observation suggests a further reaction in squalene synthesis, requiring a reducing agent. This has now been identified in the microsomes (as originally deduced; cf. Popják *et al.* 1958) and is known to be involved in the coupling of two sesquiterpenoid intermediates in the last stage of squalene synthesis (Popják, unpublished work).

Since more commonly Ca^{2+} antagonizes Mg^{2+} in enzyme reactions dependent on ATP, the activation of liver mevalonic kinase not only by Mg^{2+} but also by Ca^{2+} is unusual. The metal-ion and nucleotide requirements of mevalonic kinase are similar to those of hexokinase. However, we found that purified yeast hexokinase could not phosphorylate mevalonate and mevalonic kinase could not phosphorylate glucose. Calcium ions were ineffective with the yeast mevalonic kinase. While the liver enzyme can function only with ATP and inosine triphosphate, the yeast enzyme can use ATP and the triphosphates of guanosine, cytidine and uridine almost equally well. The pH optimum of liver mevalonic kinase is about 7·3 and that of the yeast enzyme 6·9. Both the yeast enzyme and the liver enzyme are specific for one optical enantiomorph of mevalonate; this is assumed to be the natural (+)-isomer. de Waard & Popják (1959) reported that the mevalonic acid, isolated after about 20 min. of incubating DL-[2-^{14}C]mevalonate with ATP, Mg^{2+} ions and liver-soluble enzymes, was biologically inactive. Animals injected with DL-[2-^{14}C]mevalonate excrete in their urine one-half of the radioactive dose (Gould & Popják, 1957); the mevalonate excreted in the urine is the unnatural optical enantiomorph (R. H. Cornforth, K. Fletcher & G. Popják, unpublished work).

The relative ease with which highly potent preparations of mevalonic kinase may be obtained from pig liver enabled us to prepare reasonable

quantities of 5-phospho[2-^{14}C]mevalonate. Although there can be very little doubt as to the identity of this product, nevertheless its chemical characterization has been meagre up to now.

The second phosphorylating enzyme, which uses 5-phosphomevalonate as substrate, is clearly distinct from mevalonic kinase. The enzyme preparation in which we made the preliminary studies reported here had a specific activity for the phosphorylation of mevalonate of 0·22 μmole/min./mg. of protein and for that of 5-phosphomevalonate of 0·018 μmole/min./mg. This second enzyme was activated by Mg^{2+} and not at all by Ca^{2+} ions, and could be completely removed from mevalonic kinase. The product of the action of 5-phosphomevalonic kinase on 5-phosphomevalonic acid with ATP is undoubtedly identical with compound II of Chaykin *et al.* (1958) and with the diphosphomevalonate of de Waard & Popják (1959). Chaykin *et al.* (1958) deduced by isotopic labelling that this substance must be the 5-pyrophosphate of mevalonic acid; nevertheless chemical characterization of this substance is much needed. The presence of phosphomevalonic kinase in cruder preparations of mevalonic kinase does not influence seriously the spectrophotometric assays of the latter enzyme as long as the assays are based on initial reaction rates, as the concentrations of 5-phosphomevalonate are very low at first and the activity of 5-phosphomevalonic kinase is much lower than that of mevalonic kinase.

The inhibition of mevalonic kinase by farnesoate and its saturated analogue, 3:7:11-trimethyldodecanoate, is of some interest. Inhibition of cholesterol synthesis in liver homogenates from mevalonate by farnesoate was first reported by Wright & Cleland (1957) and confirmed in this Laboratory (unpublished work). 3:7:11-Trimethyldodecanoate is an even more powerful inhibitor of cholesterol synthesis *in vitro* than is farnesoate (G. Popják & K. Clifford, in preparation) and it is somewhat surprising that it acts at such an early step in the biosynthesis. *Trans–trans*-farnesoate is formed, among other branched-chain acids, enzymically from mevalonate in liver homogenates (Popják *et al.* 1959) by the metabolism of farnesylpyrophosphate. This substance represents an alternative pathway for the metabolism of intermediates in squalene biosynthesis and may be involved in a physiological controlling mechanism of cholesterol biosynthesis (G. Popják & M. Horning, unpublished work).

Since this paper was submitted for publication, two articles appeared (Henning, Möslein & Lynen, 1959; Bloch, Chaykin, Phillips & de Waard, 1959) in which the partial purification of phosphomevalonic kinase from yeast extracts is described. Both groups of workers established beyond doubt that the diphosphomevalonate is in fact the 5-diphospho(pyrophospho)mevalonate. They also showed that 5-diphosphomevalonate yielded 5-phosphomevalonate in the presence of ADP and phosphomevalonic kinase, thereby implying the reversibility of the phosphomevalonic-kinase reaction. We did not succeed in demonstrating this.

SUMMARY

1. The enzyme which phosphorylates mevalonic acid in the 5-position, mevalonic kinase, has been partially purified from extracts of pig liver and its properties have been studied.

2. Liver mevalonic kinase is probably a sulphydryl enzyme requiring cysteine for activation under aerobic conditions. Adenosine triphosphate (or inosine triphosphate) is its coenzyme, with the Mg^{2+} ion as activator. Calcium ions, or less effectively Mn^{2+} ions, also activate the enzyme; its pH optimum is 7·3.

3. Mevalonic kinase is distinct from hexokinase, and it cannot phosphorylate a number of substances related to mevalonate. It is active on only one enantiomorph of DL-mevalonate. Potassium farnesoate, and particularly the salt of its saturated analogue, 3:7:11-trimethyldodecanoate, inhibited mevalonic kinase.

4. A large-scale preparation and purification of 5-phospho[2-^{14}C]mevalonate formed from DL-[2-^{14}C]mevalonate with mevalonic kinase is described.

5. The properties of a second enzyme, which phosphorylates 5-phosphomevalonate to the 5-diphosphomevalonate, are briefly described. This enzyme has been named 5-phosphomevalonic kinase.

6. The properties of liver mevalonic kinase are discussed in relation to a similar enzyme partially purified from yeast extracts by Tchen (1958).

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Guanosine Diphosphate Glucose and Guanosine Diphosphate Fructose from *Eremothecium ashbyii*

BY H. G. PONTIS,* A. L. JAMES AND J. BADDILEY
Department of Chemistry, King's College, University of Durham, Newcastle upon Tyne

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Guanosine diphosphate mannose was first isolated from baker's yeast (Cabib & Leloir, 1954) and has been shown to be present also in chicken oviduct (Strominger, 1955) and moulds (Ballio, Casinovi & Serlupi-Crescenzi, 1956). During the course of an investigation of the water-soluble nucleotides in the mould *Eremothecium ashbyii* we have isolated guanosine diphosphate mannose and shown that it is accompanied by two hitherto undescribed nucleotides which appear to be guanosine diphosphate glucose and guanosine diphosphate fructose. A preliminary account of this work has been published (Pontis, James & Baddiley, 1959).

EXPERIMENTAL

Materials. The guanosine and uridine phosphates were purchased from Pabst Laboratories, Wis., U.S.A. Uridine diphosphate glucose, uridine diphosphate *N*-acetylglucosamine and guanosine diphosphate mannose (GDPM) were a generous gift from Dr E. Cabib. These were supplied as barium salts and, before chromatography, the barium was removed by double decomposition with sodium sulphate.

Methods. Extinctions were measured in a Unicam SP. 500 spectrophotometer. The following analytical procedures were used: that of Fiske & Subbarow (1925) for phosphates, that of Park & Johnson (1949) for reducing power and the Roe (1934) or the indole–H_2SO_4 method (Colowick & Kaplan, 1957) for fructose. In both methods for fructose the total volume was reduced to 0·5 ml.

Paper chromatography of sugars was carried out with butanol–pyridine–water (3·0:2·0:1·5) (Jeanes, Wise & Dimler, 1951) or phenol–water (Partridge, 1948). The chromatograms were developed with the alkaline silver (Trevelyan, Procter & Harrison, 1950) or benzidine–trichloroacetic acid (Bacon & Edelman, 1951) reagents.

For the chromatography of nucleotides the $(NH_4)_2SO_4$–propan-2-ol–sodium acetate solvent (Markham & Smith, 1951) was used, and also ethanol–ammonium acetate mixtures (Paladini & Leloir, 1952). The latter solvent (pH 3·8) was used also for the chromatography of sugar phosphates. The position of the ultraviolet-absorbing substances was ascertained with a Hanovia lamp and that of phosphates by spraying with the Hanes & Isherwood (1949) molybdate reagent.

Ionophoresis on paper was performed with an apparatus similar to that described by Markham & Smith (1952), with Whatman no. 1 paper soaked in 0·05M-sodium borate, pH 9·2, for sugars (Consden & Stanier, 1952) and 0·02M-sodium acetate–HCl buffer, pH 2·0, for bases. The sugars were revealed on paper electrophoresis with the alkaline silver reagent by the modified technique described by Cabib & Leloir (1958).

In chromatographic as well as in ionophoresis experiments the position of the substances on the paper was referred to the position of an appropriate standard (ribose for

* Present address: Instituto de Investigaciones Bioquimicas, Obligado 2490, Buenos Aires, Argentina.

free sugars and adenosine for nucleotides and sugar phosphates), and the results were expressed as the ratio of the distance travelled by the unknown substance to the distance travelled by the standard substance.

Isolation of guanosine diphosphate mannose: preparation 1

Mould culture. Eremothecium ashbyii was obtained from the National Collection of Type Cultures and was cultivated as described by MacLaren (1952). Cultures were grown in 250 ml. Erlenmeyer flasks containing 50 ml. of medium at 28° under aerobic conditions (shaker) and were maintained by weekly transfer.

For the preparation of a large batch an inoculum (50 ml.) grown as described above was used to inoculate each of five 2 l. flasks containing 500 ml. of medium. After a week their contents were transferred to five 15 l. bottles each containing 10 l. of medium, where the mould was grown for a further week under conditions of forced aeration.

Preparation of the nucleotide extract. The mould culture was filtered through a 20 cm. Büchner funnel, washed with ice–water and pressed as much as possible. The mould cake (500 g. wet wt.) was cut into small pieces and mixed with 1 vol. of cold 10% trichloroacetic acid and homogenized in a Waring Blendor at full speed for 2 min. The heavy suspension was kept in the refrigerator for 1 hr. and then filtered, and the mould cake was extracted again as before three times. As soon as each acid filtrate was obtained it was extracted four times with an equal volume of ether. The acid extracts were combined, adjusted to pH 7·0 by addition of NaOH, and aerated.

Preparation of the extract for column chromatography. The acid extract (1·75 l. containing 5000 μmoles of nucleotides, calculated as uridine from E at 260 mμ) was run through a Dowex-1 X 2 (200–400 mesh) column (20 cm. × 3 cm.) in the chloride form. The column was washed with water until E at 260 mμ dropped below 0·1 and the nucleotides were eluted in a single step with 0·5 N-$CaCl_2$ in 0·01 N-HCl (720 ml.). The nucleotides eluted in this fraction amounted to 1800 μmoles (calculated as uridine). The solution was adjusted to pH 6·8 by addition of a saturated solution of $Ca(OH)_2$. Precipitation of part of the inorganic phosphate present in the extract occurred during the neutralization. The solution was filtered, concentrated *in vacuo* to a small volume, freeze-dried and the nucleotides were recovered as calcium salts (Pontis & Blumsom, 1958).

Ion-exchange chromatography. A sample of the pigment-free extract (100 ml. containing 1600 μmoles of nucleotides, calculated as uridine) was run through a Dowex-1 X 2 (200–400 mesh) column (100 cm. × 2 cm.) in the chloride form. The elution procedure of Pontis & Blumsom (1958) was used (mixing chamber containing 20 l. of 0·1 mN-HCl; reservoir containing 12 l. of 0·15 N-$CaCl_2$ in 0·01 N-HCl; $A_2/A_1 = 0·6$). A flow rate of about 2 ml./min. was used and 38 ml. fractions were collected automatically. The relevant part of the elution diagram appears in Fig. 1. The fractions corresponding to each peak were concentrated, and nucleotides were recovered as calcium salts after freeze-drying and addition of ethanol–ether (1:1) mixture. The substances corresponding to the different peaks were tentatively identified by their ultraviolet-absorption spectra. Peaks IV, V and VI were further characterized by paper chromatography of the nucleotides in the ethanol–ammonium acetate solvents. Sugars liberated by acid hydrolysis were identified by paper chromatography.

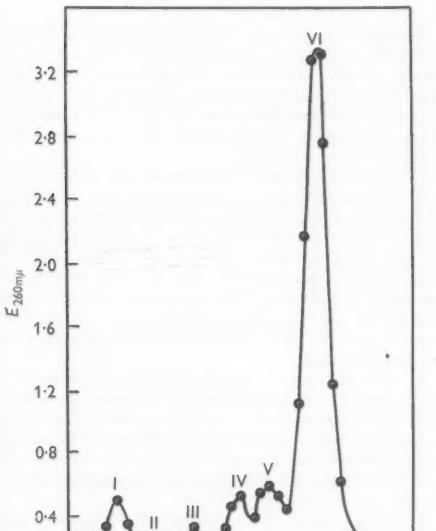

Fig. 1. Separation of mould nucleotides by ion exchange (see preparation 1 in the Experimental section). Peak I, uridine derivative; peaks II and III, flavin derivative; peak IV, uridine diphosphate *N*-acetylglucosamine, guanosine diphosphate glucose and guanosine diphosphate fructose; peak V, uridine diphosphate glucose and uridine diphosphate; peak VI, GDPM.

Isolation of guanosine diphosphate mannose: preparation 2

Preparation of the ethanolic extract. Wet mould cells (4 kg.) were kindly supplied by Mr L. R. P. Meakin of The Distillers Co. Ltd. (Biochemicals), Liverpool. This was mixed with 4 l. of ethanol and the mixture was heated with stirring until it boiled. After standing overnight (5°) it was filtered through a 20 cm. Büchner funnel with filter-aid. The extract was adjusted to pH 7·0 with alkali before chromatography. If necessary it was stored at −15°.

Ion-exchange chromatography. An ethanolic extract (2 l., containing about 14 000 μmoles of nucleotides, calculated as uridine) was run through a Dowex-1 X 2 (200–400 mesh) column (100 cm. × 3 cm.) in the chloride form. The elution procedure of Pontis, Cabib & Leloir (1957) was used with the difference that the column was washed with 0·01 N-HCl (16·9 l.) until the pH of the effluent was 2·0, before the start of the gradient elution (mixing chamber containing 22 l. of 0·02 N-NaCl in 0·01 N-HCl; reservoir containing 0·1 N-NaCl in 0·01 N-HCl). Fractions of 90 ml. were collected. The results are given in Fig. 2. The

nucleotides corresponding to peak I amounted to 248 μmoles, expressed as guanosine from E at 260 mμ (this value is corrected for the absorption due to riboflavin, which was calculated from E at 450 mμ). The nucleotides from this solution were adsorbed on to charcoal and then eluted with aqueous 50% ethanol. The flavins remained on the charcoal but the recovery of GDPM (80 μmoles) was poor.

Isolation of guanosine diphosphate mannose: preparation 3

Ion-exchange and paper chromatography. An ethanolic extract prepared as described for preparation 2 (1·5 l. containing 2400 μmoles, expressed as uridine) was run through a Dowex-1 X2 (200–400 mesh) column (25 cm. × 5 cm.) in the chloride form. After washing the column with water, nucleotides were eluted with 0·01 N-HCl (10·9 l.) followed by 0·2 N-$CaCl_2$ in 0·01 N-HCl. The second eluent was used only after E at 260 mμ had dropped below 0·1. The last fraction (7 l. containing 4250 μmoles, expressed as uridine) was adjusted to pH 6·8 by addition of a saturated solution of $Ca(OH)_2$ and the volume reduced to 600 ml. by concentration *in vacuo* at 30–35°. The calcium salts were precipitated by addition of 10 vol. of ethanol–ether (1:1) mixture and the solution was left overnight at −10°. The supernatant was decanted and the remaining suspension was centrifuged in the cold. The precipitate was washed five times with ethanol–ether mixture, twice with ether and finally dried in a desiccator. The yield was 800 mg. Before chromatography the calcium salts were suspended in water and converted into sodium salts by treatment with 0·1 N-sodium oxalate. The isolation of the mould GDPM was carried out from this solution by chromatography on Whatman no. 3 paper with ethanol–ammonium acetate (pH 3·8) for 60 hr. After chromatography the ultraviolet-absorbing zone corresponding to GDPM was cut off, the nucleotides were eluted with water and rechromatographed with the ethanol–ammonium acetate solvent (pH 7·5). After elution from the paper as before about 15 μmoles of GDPM was obtained.

Although this nucleotide preparation consisted mainly of GDPM, it contained the guanosine diphosphate glucose and guanosine diphosphate fructose. Material obtained by the methods described in preparations 2 and 3 was used in the subsequent studies on the composition of the new nucleotides. The mixture of nucleotides was chromatographically indistinguishable in both cases, and no solvent mixture was found which could resolve the three components.

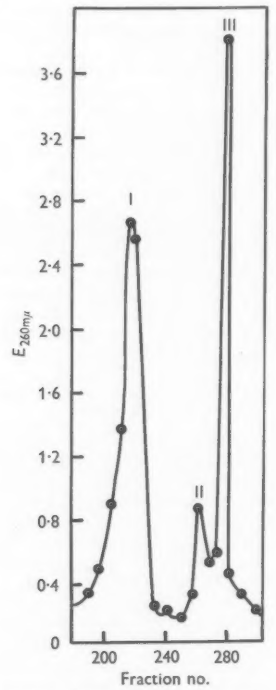

Fig. 2. Separation of the mould nucleotides by ion exchange (see preparation 2 in the Experimental section). Peak I, flavin derivative, GDPM, guanosine diphosphate glucose and guanosine diphosphate fructose; peak II, uridine diphosphate *N*-acetylglucosamine; peak III, uridine diphosphate glucose.

Degradation products of guanine nucleotide mixture

Identification of sugars. For the quantitative tests the nucleotide mixture was hydrolysed with 0·01 N-H_2SO_4 for 20 min. at 100°. The mixture was neutralized with 0·05 N-$Ba(OH)_2$ and the nucleotides were precipitated by addition of 2 vol. of 0·18 N-$ZnSO_4$ and 1 vol. of 0·37 N-$Ba(OH)_2$. After centrifuging, reducing power was determined in a portion of the supernatant.

For the determination of fructose a similar technique was used, but the portions of the supernatant were evaporated to dryness *in vacuo*, the residues were dissolved in 0·1 ml. of water and then 0·01 ml. of 1% indole in ethanol and 0·4 ml. of 75% H_2SO_4 were added. The mixture was shaken and the colour which developed after 2 hr. at room temperature was measured at 480 mμ. This colorimetric method is highly specific for fructose. Pure mannose gave an apparent fructose value of 0·6% of that given by a corresponding amount of fructose.

In order to obtain a solution of the sugars suitable for chromatography and ionophoresis, the nucleotide mixture was hydrolysed as before and the solution was treated successively with a cation-exchange and an anion-exchange resin (Dowex-50 and Amberlite IR-4 B). The resulting solution was evaporated and examined by paper chromatography and ionophoresis (Table 2).

A sample of mannose was heated with HCl under conditions similar to those described above, and then the solution was neutralized with $Ba(OH)_2$ and subjected to the $ZnSO_4$ and ion-exchange procedures. Paper chromatography of the resulting solution demonstrated the absence of fructose and glucose. Both visual and photographic techniques were employed in the examination of the paper and it is considered that the method would have demonstrated the presence of these sugars in concentrations lower than 0·5% of the mannose.

For the determination of fructose with the resorcinol–HCl reagent a hydrolysate was subjected to ionophoresis and the appropriate area of the paper was cut out and

Table 1. *Paper chromatography of nucleotides obtained by acid hydrolysis of mould–nucleotide mixture*

	$R_{adenosine}$ of u.v.-absorbing spots		
	Ethanol–ammonium acetate		Ammonium sulphate–propan-2-ol–sodium acetate
	pH 7·5	pH 3·8	
Yeast GDPM	0·13	0·11	3·47
Yeast GDPM + mould nucleotides	0·13	0·11	3·46
Mould nucleotides	0·13	0·11	3·44
Uridine diphosphate glucose	0·27	0·27	4·46
Uridine diphosphate *N*-acetylglucosamine	0·39	0·36	3·94
Guanosine diphosphate	0·03	0·05	3·42
Mould nucleotides heated for 15 min., pH 2·7 at 100°	0·03	0·06	3·43
Guanosine 5′-phosphate	0·10	0·32	3·20
Mould nucleotides heated for 3 hr., pH 2·7 at 100°	0·10	0·33	3·22
Guanosine 3′-phosphate	0·12	0·41	2·54

Table 2. *Paper chromatography and ionophoresis of sugars from mould nucleotides*

Ionophoresis was carried out on Whatman no. 1 paper, 70 cm. × 20 cm., with a potential of 600 v applied across the length of the paper during 6 hr.

	R_{ribose}		
	Paper chromatography		
	Butanol–pyridine–water*	Phenol–water	Ionophoresis with borate-buffered paper
Galactose	0·62	0·54	1·15
Glucose	0·70	0·48	1·32
Fructose	0·79	0·82	1·17
Mannose	0·83	0·61	0·94
Acetylglucosamine	0·96	1·34	0·37
Hydrolysed mould nucleotides	0·71, 0·83	0·46, 0·59, 0·81	0·94, 1·18, 1·33

* In this solvent mannose and fructose overlap.

Table 3. *Paper chromatography of the sugar phosphates obtained by the action of pyrophosphatase on mould nucleotides*

	$R_{adenosine}$ in ethanol–ammonium actate (pH 3·8)
Sugar phosphates from mould	0·59
GDPM	0·13
Glucose 1-phosphate	0·60
Glucose 6-phosphate	0·62
Fructose 1-phosphate	0·68
Fructose 6-phosphate	0·73
Fructose 1:6-diphosphate	0·29
Sucrose phosphate*	0·54

* From the data of Leloir & Cardini (1955).

eluted. Sodium borate was removed from the eluate by treatment with Dowex-50 (hydrogen form), followed by evaporation to dryness with methanol.

Hydrolysis to guanosine diphosphate and to guanosine 5′-phosphate. A sample of nucleotide mixture was hydrolysed at pH 2·7 during 15 min. at 100°. The acid was neutralized with dilute aq. NH_3 solution and the solution used directly for chromatography (Table 1).

For further hydrolysis to guanosine 5′-phosphate a sample was hydrolysed at pH 2·7 during 3 hr. at 100° and treated as described above before chromatography (Table 1). The conditions of hydrolysis were similar to those used by Cabib & Leloir (1954) for the determination of the structure of GDPM from yeast.

Action of pyrophosphatase from snake venom. To the nucleotide mixture (2 μmoles) in 2 ml. of 0·2% barbiturate buffer, pH 8·0, was added *Crotalus atrox* venom (2 mg.) and the mixture was incubated at 35° for 24 hr. The solution was treated with charcoal (about 10 mg.), centrifuged and the supernatant after concentration was used directly for chromatography. The sample was divided into two equal portions which were run in adjacent tracks on the paper. After chromatography in the ethanol–ammonium acetate solvent (pH 3·8) one track was cut out and sprayed with the reagent for phosphates. In addition to inorganic phosphate (from the hydrolysis of guanosine 5′-phosphate) a spot with $R_{adenosine}$ 0·59 was observed (Table 3). This was cut from the appropriate area of the second track and eluted. The eluted material was hydrolysed in N-HCl for 30 min. at 100° and the resulting solution was treated with Dowex-50 and Amberlite IR-4B resins and then concentrated *in vacuo*. The sugars in this solution were identified by ionophoresis as described earlier. Three sugars with the R_{ribose} values of mannose, glucose and fructose were observed.

RESULTS

Isolation of the guanosine diphosphate mannose fraction. In the initial experiments the mould was extracted with cold 10% trichloroacetic acid,

followed by extraction with ether to remove the trichloroacetic acid. As *E. ashbyii* produces large amounts of riboflavin the extract contained much riboflavin and other pigments which interfered with the normal development of the ion-exchange chromatograms, either by tailing continuously during the elution or by diminishing the capacity of the column. This difficulty was overcome by passing the extract through a short ion-exchange (Dowex-1) column, which was then washed with water, and the nucleotides were eluted as a single fraction with 0·5N-$CaCl_2$ solution (pH 2·0), followed by concentration and recovery of the nucleotides as calcium salts. The water removed the riboflavin and some pigments, and others remained adsorbed at the top of the column. The calcium salts were converted into sodium salts and chromatographed with gradient elution on a column of Dowex-1 X2 resin. Fig. 1 shows the elution pattern of the zone corresponding to the hexose-containing nucleotides.

The ultraviolet-absorption spectrum of peak IV was that of uridine. Paper chromatography in the ethanol–ammonium acetate solvents showed that this peak was mainly uridine diphosphate *N*-acetylglucosamine accompanied by traces of a slow-moving nucleotide with the same $R_{adenosine}$ as GDPM and possessing an absorption spectrum corresponding to that of guanosine. It is likely that this was GDPM, as this nucleotide is the main component of peak VI and a slight amount of cross-contamination would be expected owing to the large amount of GDPM present in these extracts (Fig. 1). However, paper chromatography of the products of mild acid hydrolysis of this minor component of peak IV revealed the presence of reducing sugars which behaved like glucose and fructose rather than mannose. Unfortunately, the amount available was insufficient for a detailed study. With the larger batches of mould the cells were extracted with ethanol and different procedures were used for the isolation of the GDPM fraction. However, it was not possible on the larger scale to separate the glucose- and fructose-containing nucleotides from GDPM.

Chromatography on Dowex-1 X2 resin in the chloride form with concave-gradient elution with $CaCl_2$, a procedure which gave good results in the initial experiments, was not successful in the large-scale experiments, possibly owing to the presence of large amounts of phosphorylated derivatives of riboflavin, which in the $CaCl_2$ system are eluted from the column in the same zone as the required nucleotides. Moreover, in the large-scale work the relative amounts of uridine diphosphate glucose and guanosine compounds in the trichloroacetic acid extract were reversed (Figs. 1 and 2), and this hindered the separation. On the other hand, gradient elution with NaCl gave a good resolution of nucleotides but the flavin-containing peak overlapped the GDPM fraction (Fig. 2). During the subsequent concentration process on charcoal most of the flavin was removed, but this technique resulted in poor recovery of the guanine nucleotides.

In order to overcome these difficulties a simpler elution procedure was coupled with paper chromatography. The ethanolic extract was run through a short Dowex-1 column, which was then washed with water followed by 0·01N-HCl, which eluted most of the monophosphates, and finally 0·2N-$CaCl_2$ in 0·01N-HCl, which eluted nucleoside diphosphates and nucleoside diphosphate sugar derivatives. After concentration the calcium salts from the last fraction were converted into sodium salts by treatment with Dowex-50 (sodium) resin or by double decomposition with 0·1N-sodium oxalate and were examined by paper chromatography in the acidic ethanol–ammonium acetate solvent. The appropriate band was cut from the paper, nucleotides were eluted and rechromatographed in the neutral ethanol–ammonium acetate solvent. Alternatively, the sodium salts were run in the $(NH_4)_2SO_4$–propan-2-ol–sodium acetate solvent, in which flavins remain at the origin.

The amounts of guanosine diphosphate glucose and guanosine diphosphate fructose obtained from the mould in the small-scale experiments, where they were free of GDPM, indicated that they were present in the mould in a concentration of about 10 μmoles/kg.

Identification of the nucleotide moiety. The absorption spectrum of the GDPM fraction from the mould was indistinguishable from that of guanosine and showed the same changes with pH. Two phosphate groups were present per molecule of guanosine and one was liberated as inorganic phosphate by hydrolysis for 20 min. in N-HCl at 100°.

Hydrolysis at pH 2·7 for 15 min. at 100° yielded guanosine diphosphate, whereas more prolonged hydrolysis (3 hr.) gave guanosine 5′-phosphate. These were identified by comparison with suitable standards in three solvent systems (Table 1). Guanine was produced by hydrolysis in N-HCl for 120 min. at 100°. This was identified by paper chromatography in the propan-2-ol–HCl solvent (Wyatt, 1951), when it had R_F 0·22. Guanine was also identified by ionophoresis in 0·02M-sodium acetate–HCl buffer, pH 2·0. The amount of material used in all these experiments was sufficiently large to enable the detection of other nucleotides and purines which may have been present in concentrations lower than 1% of the total.

Identification of the sugar moiety. Mild acid hydrolysis of the GDPM fraction liberated reducing sugars, which were submitted to paper chromatography in butanol–pyridine–water and phenol–

water, followed by development with alkaline silver or benzidine reagents. Three spots were observed with the same R_{ribose} values as glucose, fructose and mannose; similar results were obtained by ionophoresis on borate-buffered paper (Table 2). Fructose was further characterized by its reaction with resorcinol–HCl and with the indole–H_2SO_4 reagents. The absorption spectrum of the colour formed from fructose from mould nucleotides after treatment with resorcinol and concentrated acid is compared in Fig. 3 with the one obtained from pure fructose.

The reducing value of the hydrolysate was determined by Park & Johnson's (1949) method, in which the three sugars give the same colour intensity. This was used to calculate the ratio guanosine:total phosphate:reducing sugars, which was 1·04:2:1·05 (total phosphate was taken as 2). The individual sugars were determined quantitatively by eluting appropriate areas after paper ionophoresis and then estimating reducing power. The values obtained for glucose and fructose showed that together they comprised approximately 6% of the total hexose present in the GDPM from the mould.

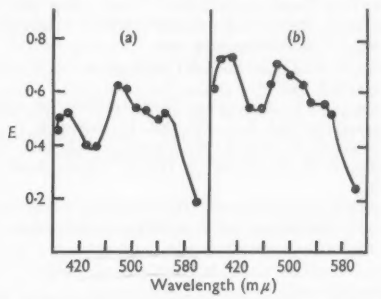

Fig. 3. Absorption spectra of the fructose moiety of guanosine diphosphate fructose and pure fructose treated with resorcinol and hydrochloric acid. (*a*) Fructose; (*b*) hydrolysed GDPM.

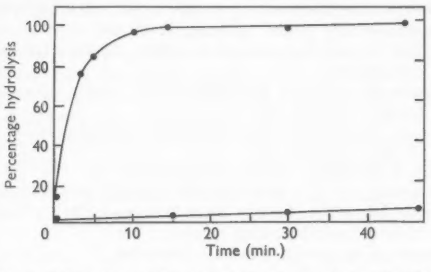

Fig. 4. Liberation of fructose from the mould nucleotides (upper curve) heated at pH 2·0, 100°. The value obtained after 20 min. was taken as 100. The lower curve is for fructose 1-phosphate.

The rate of liberation of reducing sugars from the mould nucleotide fraction is very similar to that of mannose liberation from yeast GDPM. However, small differences in the rate of hydrolysis owing to the presence of the glucose and fructose compounds would be difficult to observe on account of the large amount of mannose present. On the other hand, Fig. 4 shows specifically the rate of liberation of fructose on acid treatment. Fructose was estimated by the indole–H_2SO_4 method, which is very sensitive for this sugar even in the presence of glucose and mannose. It was shown that although the rate of liberation of fructose from the nucleotide is faster than that from fructose 1-phosphate it is somewhat slower than the liberation of mannose from GDPM, which is complete in about 5 min. at the same pH (Cabib & Leloir, 1954).

Further evidence of the nature of the linkage between the sugars and the nucleotide was obtained from the action of snake-venom pyrophosphatase. Chromatography of the products of this enzymic hydrolysis indicated the formation of a mixture of organic phosphates, which moved as a single spot in the solvent system used (Table 3). No attempt was made to isolate the guanine derivatives produced during the hydrolysis. These were removed from the reaction mixture by addition of charcoal in order to avoid interference with the chromatography of the sugar phosphates. The organic phosphates were eluted from the paper chromatogram and hydrolysed with acid, followed by paper ionophoresis on borate-buffered paper. After development with alkaline silver three spots with the same R_{ribose} values as mannose, glucose and fructose were observed.

DISCUSSION

The difficulty experienced in attempts to separate the glucose- and fructose-containing nucleotides from GDPM suggests that they possess marked structural similarities. Moreover, as all three give guanosine diphosphate or 5′-phosphate under identical conditions of hydrolysis it is concluded that both glucose and fructose are attached to guanosine through a 5′-pyrophosphate group. This is supported by the action of rattlesnake-venom pyrophosphatase, the formation of a mixture of sugar phosphates in this experiment confirming the conclusion that these were joined to the nucleoside through a pyrophosphate linkage.

Although the mixture of sugar phosphates formed by the action of the pyrophosphatase was not resolved, it is clear that this must have been a mixture of hexose monophosphates and could not

have contained a phosphate of sucrose or of any other disaccharide containing both glucose and fructose. This is apparent from a comparison of the R_F value of the sugar phosphates formed with those reported in similar solvents for phosphates of sucrose (Leloir & Cardini, 1955).

Moreover, the sugar phosphate mixture had R_F typical for hexose phosphates in 90% formic acid–ether (2:3): R_F 0·26. A sucrose phosphate would be expected to have a lower R_F in this solvent.

It is not possible on the available evidence to identify the position of the linkage between fructose and the pyrophosphate group in guanosine diphosphate fructose. The rate of liberation of fructose during acid hydrolysis of the nucleotide is comparable with the rate of hydrolysis of sucrose (Cardini, Leloir & Chiriboga, 1955), and this may suggest that the pyrophosphate is joined to position 2 in fructose. However, although fructose 1-phosphate is considerably more stable than is the linkage between nucleotide and fructose in guanosine diphosphate fructose it is not possible to eliminate position 1 from consideration. A fructose 1-pyrophosphate derivative may well be more labile than fructose 1-phosphate, this situation being analogous to that of glucose 1-phosphate and uridine diphosphate glucose (Caputto, Leloir, Cardini & Paladini, 1950).

The rate of liberation of glucose from guanosine diphosphate glucose has been studied by paper chromatography and it is concluded that this is consistent with the presence of the pyrophosphate at position 1 in glucose. This is also consistent with the ease of liberation of glucose from the sugar-phosphate mixture obtained by the action of the pyrophosphatase on the nucleotides.

The biochemical function of these nucleotides is not yet known. However, their general structure strongly suggests that they are coenzymes involved in the metabolism of glucose and fructose. It is known that a number of nucleoside–pyrophosphate–sugar compounds act as donors of their sugar moiety in the synthesis of polysaccharides. It is possible that one of the functions of guanosine diphosphate glucose and guanosine diphosphate fructose may be to act as intermediates in the synthesis of polysaccharides.

SUMMARY

1. Guanosine diphosphate mannose and two hitherto unidentified nucleotides have been isolated from the mould *Eremothecium ashbyii* by ion-exchange and paper chromatography.

2. Although the new nucleotides were not separated from guanosine diphosphate mannose they were shown to be guanosine diphosphate glucose and guanosine diphosphate fructose.

3. In guanosine diphosphate glucose a guanosine 5′-pyrophosphate residue is joined through its terminal phosphate, probably to position 1 of glucose.

4. The position of the linkage between fructose and the pyrophosphate in guanosine diphosphate fructose is not settled.

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The Ultramicro Determination of Inorganic Sulphate

By B. SPENCER
Department of Biochemistry, University of Wales, Newport Road, Cardiff

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In a previous paper (Dodgson & Spencer, 1953) a method was described for the determination of inorganic sulphate with particular reference to the study of sulphatases. The determination was a variant of the well-known benzidine method and involved treating the solution containing inorganic sulphate with an ethanolic solution of benzidine at acid pH, washing the precipitated benzidine sulphate with ethanol and estimating the benzidine moiety colorimetrically after diazotization and coupling with thymol. Stringent precautions were necessary to avoid contamination of the reaction tubes with benzidine and extreme care during manipulation was demanded. The method was therefore somewhat exacting and rather long but it has proved satisfactory, within certain limits, for various studies in this (Dodgson & Spencer, 1954; Dodgson, Lloyd & Spencer, 1957) and other laboratories (Isselbacher & McCarthy, 1959), and modifications have been introduced to meet different circumstances (Nechaeva, 1958; Slack, 1957).

To conserve laboriously prepared enzymes and other materials an attempt has been made in this Laboratory to reduce the scale of the commonly used assay techniques by a factor of usually 100 times. Many spectrophotometric determinations, such as those of arylsulphatases A and B, arylsulphatase C, β-glucuronidase, ribonucleases and non-specific phosphatases (Spencer, 1959), are readily adapted to the assay of 10 μl. samples simply by scaling down well-established methods, by the use of small reaction tubes, constriction micropipettes and microabsorption cells (cf. Lowry, 1955). However, in adapting the Dodgson & Spencer (1953) method for inorganic sulphate for use with 10 or 12 μl. samples certain changes in technique were found to be necessary. The ultramicro modification finally developed possesses considerable advantages over the original method since it is shorter, less tedious and twice as sensitive.

Bertolacini & Barney (1957) introduced a method for determining sulphate based on a new principle. It depends on the reaction in aqueous 50% ethanol between sulphate and slightly soluble barium chloranilate, leading to the formation of barium sulphate and the release of soluble chloranilic acid, which can be measured colorimetrically. The method has been modified by Lloyd (1959) for use in the presence of materials of biochemical interest, but although the procedure is simpler than that of the benzidine method of Dodgson & Spencer (1953) the sensitivity was only about one-fifth. The possibility of increasing the sensitivity of the barium chloranilate procedure and adapting it for use with small samples has now been examined.

EXPERIMENTAL

Materials. Commercial purified benzidine (May and Baker Ltd.) was recrystallized twice from 95% ethanol with the use of charcoal. Barium chloranilate was prepared and aged according to the directions of Bertolacini & Barney (1957). Powdered glass was prepared by grinding glass wool in a mortar, suspending the ground material in water and using only that part that did not settle in 10 min. under the influence of gravity. This material was washed on the centrifuge with N-HCl, water, ethanol and ether and then dried. The particle size of the powdered glass was such that in suspension it did not block the constriction micropipettes.

Apparatus. All glassware was cleaned with N-HCl; sulphuric acid mixtures must not be used for this purpose.

Benzidine sulphate precipitations were carried out in rimless centrifuge tubes of 5 cm. $\times$ 5 mm. internal diam. and tapering over the last 12 mm. to a tip of approx. 1 mm. internal diam. For the ethanolic precipitation of protein and mucopolysaccharides in both the benzidine and barium chloranilate methods rimless tubes of 5 cm. $\times$ 3 mm. internal diam. and tapering only over the last 5 mm. were preferred. These tubes were also suitable for the reaction between barium chloranilate and sulphate-containing solutions. All tubes were readily made from glass tubing.

Solutions were delivered with Lang–Levvy constriction micropipettes (Lowry, Roberts, Leiner, Wu & Farr, 1954). Owing to the low surface tension of ethanol, pipettes used for ethanolic solutions require rather smaller orifices and constrictions than are normal for aqueous solutions and should be calibrated (Lowry *et al.* 1954) with ethanol. Contents of the small tubes were mixed by 'buzzing' against a flattened nail rotating at speeds from 300 to 1000 rev./min., depending on the degree of agitation required. Tubes were sealed with Parafilm (A. Gallenkamp and Co. Ltd.) and centrifuging was carried out with multiplace bored-out aluminium blocks. Spectrophotometric readings were made in cuvette-type microcells (Lowry & Bessey, 1946) in a Hilger Uvispek spectrophotometer with the microcell adaptor. Practical details for the construction, use and cleaning of the apparatus of the type mentioned above have been reviewed by Lowry (1955).

RESULTS

Benzidine method

Absorption spectrum of benzidine. In the benzidine method used previously (Dodgson & Spencer, 1953) the precipitated benzidine sulphate was measured colorimetrically after diazotization and subsequent coupling with thymol. The azo dye formed shows a broad peak at 510 mμ with ϵ_{510} 56 000 (Fig. 1). However, the factor which limits the sensitivity of the benzidine method is not the final colorimetric measurement but the inability to precipitate small amounts of benzidine sulphate. Some decrease in sensitivity in the measurement of the precipitated benzidine sulphate can therefore be accepted. Benzidine in N-HCl shows maximum absorption at 248 mμ with ϵ_{248} 19 200 (Fig. 1). Absorption at $\lambda_{max.}$ obeys the Lambert–Beer law up to the highest value tested, E 1·5, and measurements at 248 mμ in 1 cm. cells have proved satisfactory for the estimation of precipitated benzidine sulphate.

Determination of sulphate in aqueous solution. To 10 μl. of the sulphate-containing solution in a 5 mm. internal diam. tube were added 10 μl. of 20% (w/v) trichloroacetic acid (containing 10 mg. of powdered glass/ml. and 10 μg. of K_2SO_4/ml.) and 50 μl. of freshly prepared 1% (w/v) benzidine in ethanol. The tube was capped and the contents were mixed by 'buzzing'. After standing in the refrigerator (2°) for not less than 1 hr. the tube was centrifuged at 3000 *g* for 10 min. at 2°. The supernatant was removed by suction with a fine tube with a bent tip. With care, all the visible fluid can be removed without disturbing the precipitate. About 100 μl. of ethanol from a 120 μl. pipette was added into the top of the tube, which was turned during the addition to wash down its walls. The tube was 'buzzed' until the precipitated powdered glass was completely and evenly suspended and then the 20 μl. of ethanol remaining in the pipette was added to wash down the walls of the tube. The tube was capped, centrifuged and the supernatant removed as before. The precipitated benzidine sulphate was dissolved by 'buzzing' with 100 μl. of N-HCl. After a quick centrifuging to throw down the powdered glass, the absorption of the benzidine at 248 mμ was measured in 1 cm. microcells. Blank determinations, in which water was substituted for the sulphate-containing solution, were carried out. Both blank and test determinations were made in duplicate or triplicate. The amount of sulphate in the original sample was calculated from the difference in test and control absorption readings, the volume of the final solution and ϵ_{248} 19 200 for benzidine.

Reasonable care is required during these operations if contamination of the tubes with benzidine is to be avoided. Only the tip of the pipette used for benzidine addition should come into contact with the stock benzidine solution and this pipette should be cleanly inserted to near the bottom of the tube before delivery of the solution. 'Buzzing', at all stages, should be vigorous enough to suspend the glass powder evenly but not so strong as to throw the solution more than about half-way up the tube.

The above directions were suitable for 10 μl. samples containing 20–400 μmg. of SO_4^{2-} ion, giving E 0·040–0·800. For samples containing over 400 μmg. of SO_4^{2-} ion the washed benzidine sulphate should be dissolved in a proportionately greater volume of N-HCl. The procedure can be adapted to the estimation of SO_4^{2-} ion in 20 μl. samples by doubling the volume of all the reagents used, and this scale has been preferred by some users of the method. The size of tube remains the same. The method has also been used for 100 μl. samples of sulphate-containing solution by working in 15 ml. tapered centrifuge tubes. The method and precautions are as described before but all volumes must be increased ten times. On this scale the final solution for light-absorption measurement is 1 ml. and readings can be made in normal 1 cm. cells by masking the appropriate spectrophotometer slit with a piece of opaque paper to restrict the beam of light so that it passes under the meniscus of the liquid in the cell.

An alternative procedure for the measurement of the precipitated benzidine sulphate, recommended for use only when a u.v. spectrophotometer is not available, is carried out as follows. The benzidine sulphate was dissolved in HCl–$NaNO_2$ mixture (3 vol. of N-HCl and 1 vol. of 0·1% $NaNO_2$, added together immediately before use). The diazotized benzidine was coupled with thymol by the addition, with immediate and rapid mixing, of 0·5% thymol in 7·5% (w/v) NaOH containing 10% (w/v) of Na_2SO_3. The absorption of the coloured product was measured at 510 mμ. The HCl–$NaNO_2$ mixture and the thymol solution should be added in the ratio 4 : 5, in volumes calculated from the value ϵ_{510} 56 000 for the coloured product and the approximate sulphate concentration of the original sample. For example, when the coloured product was contained in a final volume of 360 μl., i.e. 160 μl. of HCl–$NaNO_2$ and 200 μl. of thymol solution, the readings

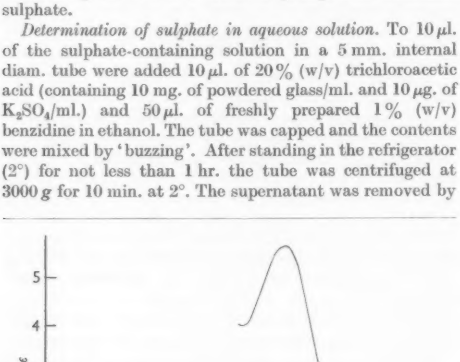

Fig. 1. Absorption spectra of azo dye (—), benzidine (. . .) and chloranilic acid (- - -). Azo dye was prepared by diazotization of benzidine followed by coupling with thymol under the conditions described in the text. The benzidine was dissolved in N-HCl. For the spectrum in the region 350–600 mμ, chloranilic acid was dissolved in 66·7% (v/v) ethanol containing 0·117 M-acetate, pH 4·0; these are the conditions used in the barium chloranilate method for large concentrations of SO_4^{2-} ion. For the region 200–350 mμ the chloranilic acid was dissolved in 6·1% (v/v) ethanol containing 0·46 M-acetate, pH 4·0; these are the conditions used in barium chloranilate method with low concentrations of SO_4^{2-} ion. The right-hand ordinate gives 10^{-3} ϵ values for chloranilic acid between 350 and 360 mμ; the left-hand ordinate gives 10^{-4} ϵ values for the remaining three curves.

were E 0·081–0·810 for the range 50–500 μmg. of SO_4^{2-} ion/10 μl. of original sample. This volume of liquid was large enough for the absorption at 510 mμ to be read in normal 1 cm. cells by using a suitably masked spectrophotometer as previously described. The Na_2SO_3 was added to the thymol solution to prevent the formation of a brown oxidation product which was formed, in its absence, at the interface between the HCl–$NaNO_2$ solution and the thymol before the two solutions could be mixed. This trouble was not encountered when 15 ml. centrifuge tubes and larger volumes were used as in the original method (Dodgson & Spencer, 1953), since considerable mixing occurred in these wider tubes during the addition of the thymol solution.

Determination of sulphate in biological materials. To 12 μl. of the tissue suspension or other material contained in a 3 mm. internal diam. tapered tube was added 48 μl. of ethanol and the tube was capped, 'buzzed' and centrifuged at approx. 3000 g for 10 min. A 50 μl. portion of the supernatant was pipetted into a 5 mm. internal diam. tapered tube and 10 μl. of 20% (w/v) trichloroacetic acid (containing 10 mg. of powdered glass and 10 μg. of K_2SO_4/ml.) and 10 μl. of a freshly prepared ethanolic 5% (w/v) benzidine were added. The tube was kept at 0° for at least 1 hr. and the benzidine sulphate precipitated, washed and determined as before. Control determinations, in which the tissue suspension was replaced by the same amount of suspension medium, were carried out. In the calculation, allowance must be made for a 2% contraction in volume when 48 μl. of ethanol is added to 12 μl. of aqueous solution.

Recovery of added sulphate. Previous experience with the benzidine method and 1 ml. samples had shown that under certain conditions the recovery of added sulphate was not quantitative (Dodgson & Spencer, 1953). The main sources of error were the incomplete precipitation of small amounts of sulphate and interference at the precipitation stage by various anions and cations. The ultramicro modification was found to be subject to the same errors. In pure aqueous solution, recovery from samples containing less than 75 μmg. of SO_4^{2-} ion as K_2SO_4/10 μl. was low. Good recoveries (±4%) were obtained by adding 100 μmg. of K_2SO_4 to both the tests and controls and this procedure was incorporated into the standard method. Recovery of 100 μmg. of SO_4^{2-} ion from 12 μl. of 0·5 M-sodium acetate–acetic acid containing various sulphatase substrates or tissue suspensions was satisfactory (Table 1). Variations of the pH of the acetate buffer between pH 3·5 and 7·0 did not affect the recoveries. In acetate buffer, pH 5·0, NaCl, KNO_3, $MgCl_2$, adenosine triphosphate, glucose at a concentration of 0·01 M and $FeCl_3$ and $CaCl_2$ at a concentration of 0·001 M did not affect the recovery of 122 μg. of SO_4^{2-} ion/12 μl. of sample. The cations Fe^{3+} and Ca^{2+} at a concentration of 0·01 M considerably depressed the precipitation of benzidine sulphate and recovery of added sulphate was less than 50%. The method was inoperable in the presence of traces of barium. Low recoveries of sulphate were observed in the presence of KH_2PO_4 at concentrations exceeding 0·03 M.

Barium chloranilate method

Absorption spectrum of chloranilic acid. The sensitivity of the barium chloranilate method is governed by the low absorption maximum of chloranilic acid in the visible region, although recently Lloyd (1959) has increased the sensitivity by making measurements at 350 mμ. The spectrum of chloranilic acid at pH 4·0 shows absorption maxima at 530, 327·5 and 235 mμ with ϵ 220, 24 300 and 17 400 respectively (Fig. 1). A 100-fold increase in sensitivity in estimating chloranilic acid can therefore be obtained by making absorption measurements at 327·5 rather than 530 mμ. The Lambert–Beer law is obeyed at 327·5 mμ up to E 1·5.

Determination of sulphate in aqueous solution. To 10 μl. of the sulphate-containing solution in a 3 mm. internal diam. tapered tube was added 40 μl. of ethanol. After mixing by 'buzzing', 5 μl. of 0·5 M-acetate buffer, pH 4·0, containing approx. 72·5 μg. of K_2SO_4/ml., was added and the solutions were mixed. A further 5 μl. of 0·5 M-acetate buffer, pH 4·0, containing 20 mg. of barium chloranilate/ml., was then added, the tube was capped with Parafilm and 'buzzed' vigorously at minute intervals over 10 min. The mixture was centrifuged at 3000 g for 10 min. and a 20 μl. portion of the supernatant was diluted with 200 μl. of 0·5 M-acetate buffer, pH 4·0. The absorption of the liberated chloranilic acid in the diluted solution was measured at 327·5 mμ in 1 cm. cells. Determinations were made in duplicate with duplicate controls in which water was substituted for the sulphate-containing sample. These directions were suitable for use with 10 μl. samples containing 0·25–2·5 μg. of

Table 1. *Recovery of added potassium sulphate from biological materials by the benzidine method*

Material	Concn. in the sample (%, w/v)	SO_4^{2-} ion added (μmg./12 μl.)	SO_4^{2-} ion recovered (μmg.)
Acetone-dried human liver	0·5	100·7	98·3
Fresh rat liver (wet wt.)	1·0	100·7	97·7
Fresh rat kidney (wet wt.)	2·0	100·7	98·0
Acetone-dried *Proteus vulgaris*	0·5	100·7	98·9
Acetone-dried *Alcaligenes metalcaligenes*	0·5	100·7	102·3
Acetone-dried *Charonia lampas*	0·5	100·7	99·5
Taka-diastase in 0·5 M-acetate buffer, pH 3·5	0·5	100·7	98·3
Taka-diastase in 0·5 M-acetate buffer, pH 5·0	0·5	100·7	97·9
Taka-diastase in 0·5 M-acetate buffer, pH 6·0	0·5	100·7	99·6
Chondroitin sulphate C (shark cartilage)	0·15	122·3	118·8
Chondroitin sulphate A (ox cartilage)	0·15	122·3	118·7
Potassium *p*-nitrophenyl sulphate	0·05 M	122·3	120·0
Potassium glucose 6-sulphate	0·02 M	122·3	119·4
Choline sulphate	0·05 M	122·3	118·4
Sinigrin	0·05 M	122·3	121·1

SO_4^{2-} ion giving E 0·096–0·960. With higher sulphate concentrations, 5·0–25·0 μg. of SO_4^{2-} ion/10 μl. of sample, 10 μl. of 0·5 M-acetate containing 10 mg. of barium chloranilate/ml. was added to the sample plus ethanol mixture instead of the two 5 μl. additions as detailed above. Measurements were then made on the undiluted final supernatant at 530 mμ. The sulphate contents of the samples were calculated from the dilutions made by using the appropriate molecular extinction coefficient for chloranilic acid (see above). The procedures were readily adapted to either a 10- or 50-fold increase in scale by using 15 ml. tapered centrifuge tubes.

Determination of sulphate in biological materials. A 12 μl. sample of the tissue suspension or other material was deproteinized with 48 μl. of ethanol as described for the benzidine method. To 50 μl. of the deproteinized solution were added 5 μl. of the acetate–K_2SO_4 solution plus 5 μl. of acetate–barium chloranilate suspension or 10 μl. of the acetate–barium chloranilate suspension, depending on the approximate sulphate content of the original sample. The further treatment was in the manner described for the determination of sulphate in aqueous solutions.

Recovery of added sulphate. When the reaction mixture was composed of 10 μl. of the sulphate-containing mixture, 40 μl. of ethanol and 10 μl. of 0·5 M-acetate buffer, pH 4·0, containing 10 mg. of barium chloranilate/ml., the recovery of 5·0–25·0 μg. of SO_4^{2-} ion/10 μl. of sample was 100 $\pm$ 2 %, but in the range 0·25–2·5 μg. of SO_4^{2-} ion/10 μl. of sample the recovery was low. Fig. 2 shows that at least 0·1 μg. of SO_4^{2-} ion/10 μl. of sample must be present before release of chloranilic acid from barium chloranilate starts. This limiting effect was readily corrected, as detailed in the method, by adding extra SO_4^{2-} ions to the sample plus ethanol, in both test and controls, before addition of the barium chloranilate. With this procedure there was a straight-line relationship between concentration of SO_4^{2-} ion, in the range 0·25–2·5 μg. of SO_4^{2-} ion/10 μl. of sample, and the difference between test and control readings at 327·5 mμ (Fig. 2). With the higher sulphate concentrations the error introduced by the limiting-concentration effect was not significant and there was no need to add extra sulphate.

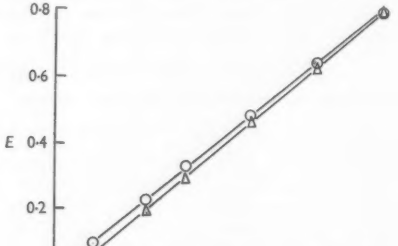

Fig. 2. Relationship between chloranilic acid liberated and sulphate concentration. The chloranilic acid released from barium chloranilate by 10 μl. samples of known amounts of SO_4^{2-} ion under the conditions described in the text was measured at 327·5 mμ. △, Normal 10 μl. samples; ○, samples containing an extra 200 μmg. of SO_4^{2-} ion.

Unsuccessful attempts were made to estimate 25–251 μmg. of SO_4^{2-} ion/10 μl. of sample by making absorption readings at 327·5 mμ on the undiluted supernatant. With this procedure it was found that the 200 μmg. of SO_4^{2-} ion/10 μl. of sample, added to avoid the limiting-concentration effect, resulted in the control readings being very high compared with the difference between the test and control. Furthermore, the exchange of barium between chloranilic acid and SO_4^{2-} ion did not appear to proceed to completion and with amounts of SO_4^{2-} ion of the order of 100 μmg./10 μl. low recoveries of approx. 70 % were obtained. An increase in recovery of SO_4^{2-} ion to 85 % was made by using aqueous ethanol in the procedure so as to obtain an ethanol concentration of 35 % (v/v) in the reaction mixture (instead of the usual 66·5 %). Unfortunately there was also a large increase in control reading under this circumstance and further lowering of the ethanol concentration was not feasible.

The recovery of 0·25–2·5 μg. of SO_4^{2-} ion/10 μl. of sample was tested in the presence of a number of anions and cations, sulphatase substrates and tissue and enzyme preparations. In general, the findings of Lloyd (1959) were repeated except that recovery of low sulphate concentrations was 5–15 % too high in the presence of 0·02 M-PO_4^{3-} ions. At concentrations of PO_4^{3-} ion below 0·01 M no interference was observed.

Application of the benzidine method

Chondrosulphatase of Charonia lampas. Extracts of the tropical marine-mollusc *C. lampas* have been reported to contain both a chondrosulphatase and a chondroitinase (Soda & Egami, 1938). Before attempting to extend this observation, samples of acetone-dried *Charonia* liver, obtained from Professor F. Egami, were tested for their ability to degrade chondroitin sulphate by incubation of the preparations with chondroitin sulphate and measurement of the release of inorganic sulphate and reducing activity by the benzidine method and an ultramicro modification (B. Spencer, unpublished work) of the Somogyi (1952) method respectively.

To 50 μl. of a 4 % (w/v) suspension of acetone-dried *Charonia* liver in 0·5 M-acetate buffer, pH 5·0, was added 50 μl. of water or 50 μl. of a solution containing 2 mg. of sodium chondroitin sulphate/ml. and the mixture incubated at 38°. Samples (12 μl.) of the incubation mixture were removed at zero time and after incubation for 3 hr., and SO_4^{2-} ion and reducing activity were determined. The experiment was repeated with the centrifuged supernatant of the *Charonia* suspension.

Unmeasurably large amounts of reducing activity were liberated during 3 hr. incubation of the *Charonia* suspension in the absence of added substrate. This was attributed to the enzymic degradation of endogenous insoluble polysaccharides, for when the supernatant of the centrifuged *Charonia*

preparation was used the release of reducing material was much smaller and readily measured. No extra release of reducing activity was observed in the presence of chondroitin sulphate from ox or shark cartilage. There was a slight liberation of SO_4^{2-} ions from endogenous substrates in the *Charonia* suspension amounting to 5 μg./g. of *Charonia* powder/3 hr., but no extra SO_4^{2-} ion was released from the chondroitin sulphate preparations during the incubation with the whole suspension of *Charonia* powder or the centrifuged supernatant. The experiment was repeated at pH 4·0 and 6·0 but no significant liberation of sulphate or reducing activity from the chondroitin sulphates was observed during 3 hr. incubation. The lack of chondrosulphatase activity in three different *Charonia lampas* preparations has been confirmed by other workers in this Laboratory.

Acid hydrolysis of choline sulphate. During an investigation of the role of choline sulphate in the sulphur metabolism of fungi (Spencer & Harada, 1959) it became necessary to know the conditions necessary for complete hydrolysis of choline sulphate by acid.

The acid solution of choline sulphate (0·5 mg./ml.) was heated under a finger reflux condenser in a boiling-water bath and 1 ml. samples were removed at various times. To each sample was added 2 μl. of bromocresol green-indicator solution; the mixture was neutralized with NaOH and made up to 2 ml. The SO_4^{2-} ion in 10 μl. of the diluted samples was estimated by the benzidine method.

Complete hydrolysis of choline sulphate took place in 25 min. in 2N-HCl at 100°. In 0·3N-HCl for 30 min. at 100°, the conditions used by Raistrick & Vincent (1948) for the hydrolysis of ester sulphates in fungi, only 23 % hydrolysis had occurred.

DISCUSSION

The small-scale modification of the benzidine method for sulphate estimation offers certain advantages over the original method (Dodgson & Spencer, 1953). In addition to the saving of materials and the application to small sample volumes the modification is twice as sensitive as, and very much more rapid than, the larger-scale method. The number of manipulations is halved and in practice it has proved relatively simple to gain proficiency in its use. Apart from the applications already given, the modified method is being used in this Laboratory in the study of the glycosulphatase of *Littorina littorea* (K. S. Dodgson, unpublished work), for the analysis of sulphate esters isolated in small amounts by paper chromatography and for the determination of SO_4^{2-} ions and ester sulphates in fungal mycelia.

The success of benzidine methods for sulphate estimation depends largely on the efficient removal of unprecipitated benzidine. In the small-scale method all benzidine-containing mixtures were restricted to the bottom half of the reaction tube and no serious contamination of the upper parts of the tube occurred. In the larger-scale method (Dodgson & Spencer, 1953) the benzidine-containing solutions were poured from the tube and much of the subsequent manipulation was concerned with the removal of the contaminating benzidine from the mouth and walls of the reaction tube. Of course, some benzidine was left behind in the tubes in the small-scale method but with a little practice in the technique the differences between the amounts of benzidine remaining in the individual tubes, as shown by the differences between the absorption readings of duplicate determinations, were not significant. Although no advantages were gained (cf. Nechaeva, 1958), it was noted that some of the remaining benzidine can be removed and the blank values lowered a little by a second washing of the precipitated benzidine sulphate with 120 μl. of ethanol. The recovery of SO_4^{2-} ion by this procedure was only 96 $\pm$ 4 % (cf. Dodgson & Spencer 1953).

The measurement of the benzidine of the precipitated benzidine sulphate by direct spectrophotometric measurement involved only one pipetted addition, whereas three additions were required for the development of the azo dye in the larger-scale method. No interference in the absorption at 248 mμ was observed with the variety of biological materials listed in Table 1. Proteins were, of course, precipitated by ethanol and removed by centrifuging and other materials which absorb in the 248 mμ region and which were soluble in the aqueous 80 % ethanol were effectively washed away in the subsequent manipulations.

The procedure adopted for the determination of SO_4^{2-} ion by the barium chloranilate method was slightly different from that of Lloyd (1959) and two extra steps were introduced: the addition of a small amount of extra SO_4^{2-} ion and the dilution of the final supernatant before making absorption readings at 327·5 mμ. In the method of Lloyd (1959) no absolute reference was made to the chloranilic acid liberated from barium chloranilate by SO_4^{2-} ion and values for concentrations of SO_4^{2-} ion were obtained by reference to a standard curve obtained with known amounts of SO_4^{2-} ion in the range 25–400 μg. of SO_4^{2-} ion/ml. of sample. The requirement for a limiting concentration of 10 μg. of SO_4^{2-} ion/ml. of sample before exchange of barium between chloranilic acid and SO_4^{2-} ion takes place was also found to apply to Lloyd's method, but with the use of the standard curve the

accuracy of the method was not affected. However, in both Lloyd's method and the present small-scale method the limiting concentration of SO_4^{2-} ion was found to vary slightly in the presence of biological materials and various ions, particularly PO_4^{3-} and Mg^{2+}. With concentration of 110 μg. of SO_4^{2-} ion/ml. of sample the effect of this variation influenced the recovery by less than 3% (cf. Lloyd, 1959), but with lower sulphate values, such as 35 μg. of SO_4^{2-} ion, the error was sometimes greater than 10%. These discrepancies were readily corrected by the addition of extra SO_4^{2-} ion, to both test and controls.

The dilution of the final supernatant before measuring the absorption at 327·5 mμ was necessary because of the high $\epsilon_{327\cdot5}$ of chloranilic acid. The pitfalls in making absorption readings at a wavelength where the absorption curve is steeply ascending are well recognized and the measurement of chloranilic acid at 350 mμ (Lloyd, 1959) is not recommended (cf. Fig. 1).

It is disappointing that the sensitivity of the barium chloranilate procedure has been increased only by a factor of 2 over the procedure of Lloyd (1959) and that it does not seem possible to determine less than 25 μg. of SO_4^{2-} ion/ml. by this method. The benzidine and barium chloranilate methods therefore remain complementary, the small-scale benzidine method being the choice for sensitivity and for use in the presence of small amounts of bivalent cations and the present barium chloranilate method or method of Lloyd (1959) being the choice for rapidity and convenience and for use in the presence of PO_4^{3-} ions.

SUMMARY

1. A scaled-down version of the benzidine method of Dodgson & Spencer (1953) has been developed for the estimation of 20–400 μmg. of SO_4^{2-} ion in 10 μl. samples. The precipitated benzidine sulphate is estimated by direct spectrophotometric measurement of benzidine at 248 mμ. Modifications of the method for use with higher concentrations of SO_4^{2-} ion and for 20 and 100 μl. samples are described.

2. The scaled-down version is twice as sensitive as, and very much more rapid than, the original method.

3. By means of the benzidine method potassium sulphate can be quantitatively recovered in the presence of various tissue suspensions, sulphatase substrates and small amounts of certain inorganic ions.

4. The barium chloranilate method has been scaled down for use with 10 μl. samples containing 0·25–2·5 or 2·5–25 μg. of SO_4^{2-} ion.

5. With the benzidine method it was observed that three different samples of acetone-dried liver of *Charonia lampas* failed to liberate SO_4^{2-} ion from chondroitin sulphate.

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Studies on Carbohydrate-Metabolizing Enzymes

3. YEAST BRANCHING ENZYME*

BY ZEENAT H. GUNJA, D. J. MANNERS AND KHIN MAUNG
Department of Chemistry, University of Edinburgh

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The classical researches of Hanes, Peat and their co-workers (for reviews, see Barker & Bourne, 1953; Whelan, 1958) have established that two distinct enzymes are involved in the synthesis of amylopectin from α-D-glucosyl phosphate. Thus P-enzyme (potato phosphorylase) action results in the formation of linear amylose-type molecules which are converted into amylopectin by Q-enzyme. Alternatively, the combined action of P- and Q-enzymes on α-D-glucosyl phosphate also yields a branched polysaccharide, although Q-enzyme alone has no action on the glucoside.

In the animal kingdom a related enzyme system may be partly responsible for glycogen synthesis. Muscle phosphorylase synthesizes amylose-type chains from α-D-glucosyl phosphate and the concurrent action of this enzyme and an enzyme preparation (branching factor or supplementary enzyme) from heart or liver extracts gave a glycogen-type polysaccharide, since the latter gave a red–brown coloration with iodine and, unlike amylose, could function as a primer for muscle phosphorylase (Cori & Cori, 1943). In general, however, the branching enzymes from animal tissues have not been highly purified and the products of enzyme action have not been rigidly characterized.

Glycogen is one of the reserve polysaccharides of brewer's yeast (Manners & Khin Maung, 1955*a*); hence, extracts of this yeast should provide a source of branching enzyme. This enzyme was first encountered as an impurity in yeast-phosphorylase preparations (unpublished work), which, acting on α-D-glucosyl phosphate, synthesized a branched polysaccharide which had a β-amylolysis limit of 44% and gave a red–brown stain with iodine.

This paper describes some of the properties of partly purified yeast branching enzyme and its action on amylose. Furthermore, since yeast glycogen contains 7–8% of 1:6-glucosidic linkages, it was of interest to examine the action of yeast branching enzyme on an amylopectin containing only 4–5% of branch points. Preliminary accounts of some of this work have been published (Manners & Khin Maung, 1956; Gunja & Manners, 1959).

* Part 2: Archibald & Manners (1959).

METHODS AND MATERIALS

Analytical methods. Reducing sugars and protein N were determined by the methods of Somogyi (1952) and Robinson & Hogden (1940) respectively. Enzyme digests containing isoamylase were deproteinized by Nelson's (1944) method. Paper chromatography was carried out as described by Manners & Khin Maung (1955*a*) and Archibald & Manners (1959).

Iodine staining. Polysaccharide sample (0·5–1·0 ml.) was stained with iodine solution [0·2% of I_2 in 2% (w/v) KI; 0·5–1·0 ml.] in a total volume of 50–100 ml. The solution also contained 1 drop of 5N-HCl. The absorption value of the polysaccharide–iodine complex [for definition see Bourne, Haworth, Macey & Peat (1948)] was measured (*a*) by a Hilger Spekker photoelectric absorptiometer in 1 or 4 cm. cells with an Ilford no. 608 (approx. 680 mμ) filter (amylose or starch) or no. 603 (approx. 490 mμ) filter (waxy-maize starch) against an iodine–water blank, or (*b*) with a Unicam SP. 500 spectrophotometer with 1 cm. cells against an iodine–water blank. Amylose, starch and amylopectin solutions were measured at 630, 600 and 540 mμ respectively.

Phosphorylase activity. The conditions of Green & Stumpf (1942) were used; inorganic phosphate was measured by the method of Fiske & Subbarow (1925).

Analysis of polysaccharides

Glucose content. The polysaccharides (0·1%, w/v) were hydrolysed with 2N-H_2SO_4 at 100° for 2 hr. and the reducing-sugar content (as glucose) was determined with the Somogyi reagent. Analytical figures are based on the observed glucose contents.

β-Amylolysis limit. Polysaccharide (25 mg.), 0·2M-sodium acetate buffer (pH 4·6; 4 ml.) and β-amylase (approx. 1250 units) were incubated in a total volume of 25 ml. at 35° for 24 hr. The maltose content of a portion (3 or 5 ml.) was then determined. Under these conditions enzyme action was 93% complete in 4 hr. and the maltose content after 24 and 48 hr. was identical (Liddle, 1956).

β-Amylolysis limit after incubation with isoamylase. Polysaccharide (25 mg.), 0·2M-sodium acetate buffer (pH 6·0; 4 ml.), isoamylase (50 mg.) and water (7 ml.) were incubated at 20° for 24 hr. The digest was heated (3 min. at 98°), cooled and β-amylase solution (approx. 1250 units) and water (to 25 ml.) were added. After 24 hr. at 35°, the maltose content of the digest was determined after deproteinization of a sample.

Simultaneous action of β-amylase and isoamylase. Polysaccharide (20–25 mg.), 0·2M-sodium acetate buffer (pH 6·0; 4 ml.), isoamylase (40 mg.) and β-amylase (1000 units) were incubated for 24 hr. at 20° in a total volume of

25 ml. After deproteinization of a sample (3 ml.) the maltose content was measured.

α-Amylolysis limit. Polysaccharide (30 mg.), 0·1 M-NaCl solution (10 ml.), freeze-dried α-amylase (2·5 mg.) and water (to 50 ml.) were incubated at 35° for 24 hr. Portions (5 ml.) were examined for reducing sugar (expressed as apparent percentage conversion into maltose, P_M).

Limiting viscosity number. By means of a modified Ubbelohde viscometer, the specific viscosity, $\eta_{sp.}$, at 25±0·02° of four concentrations of the polysaccharide in 0·1 M-NaCl or M-KOH was determined. The limiting viscosity number, $[\eta]$, was obtained by extrapolation of $\eta_{sp.}/c$ against c to zero concentration (c as g./ml.).

Enzyme preparations

Pressed brewer's yeast (*Saccharomyces cerevisiae*) was kindly supplied by Wm. Younger and Co. Ltd. It was washed with water, dried at 35° in a current of air and stored in air-tight bottles at 0°. Isoamylase was prepared by the method of Manners & Khin Maung (1955*b*) and Gunja, Manners & Khin Maung (in preparation). α- and β-Amylase were as described by Liddle & Manners (1957); soya-bean β-amylase was also used (Manners & Khin Maung, 1955*a*).

Substrates

Amylose. Various samples, made by the fractionation of potato starch with butanol (H. Baum & G. A. Gilbert, cited by Whelan, 1955), aluminium hydroxide (Bourne, Donnison Peat & Whelan, 1949) or thymol and butanol (Greenwood & Robertson, 1954), were used.

Amylopectin. Two samples of amylopectin were prepared by the thymol fractionation of potato starch (Greenwood & Robertson, 1954). Malted-barley amylopectin was kindly provided by Dr G. O. Aspinall (Aspinall, Hirst & McArthur, 1955). Waxy-maize starch was a commercial sample.

Glycogen. Glycogens isolated from brewer's yeast (Manners & Khin Maung, 1955*a*), rabbit muscle (Liddle & Manners, 1957) and horse-diaphragm muscle (Lawrie, Manners & Wright, 1959) were used. Floridean starch was the sample II described by Fleming, Hirst & Manners (1956).

β-Dextrins. Amylopectin β-dextrin and glycogen β-dextrin were prepared by the prolonged action of β-amylase on waxy-maize starch and *Mytilus edulis* VI glycogen (Liddle & Manners, 1957), followed by dialysis (to remove maltose and inorganic material) and isolation by precipitation with ethanol or freeze-drying.

Preparation of yeast branching enzyme

The starting material was air-dried brewer's yeast. The following procedure has given preparations of yeast branching enzyme of satisfactory activity, although with different batches of yeast the proportion of contaminating enzymes (phosphorylase, isoamylase, isomaltase and maltase) has varied. Dried yeast (100 g.) was extracted with 0·1 M-$NaHCO_3$ solution (500 ml.) at 35° for 2 hr., and the extract centrifuged at 0°. To the solution (about 350 ml.) an equal volume of cold saturated $(NH_4)_2SO_4$ solution was added. The precipitate was collected by centrifuging at 1° and dissolved in 0·1 M-sodium citrate buffer (pH 6·6; 100 ml.). Aq. 50% (v/v) ethanol–0·01 M-sodium citrate buffer, pH 6·8, was added at −5° to give an ethanol concentration of 25%.

The precipitate was removed and the ethanol concentration of the supernatant solution was increased to 30%. This precipitate was collected and triturated with 0·1 M-sodium citrate buffer (pH 6·8; 40 ml.) for 30–60 min. Insoluble material was removed by centrifuging and the solution treated with an equal volume of cold saturated $(NH_4)_2SO_4$ solution. The precipitate was collected, dissolved in 0·1 M-sodium citrate buffer (pH 6·8; 20 ml.) and freeze-dried. Yield 2·0–2·6 g.; N content 1·6–2·3%. The material was free from iodophilic polysaccharide.

A digest containing amylose (2·5 mg.), 0·2 M-sodium citrate buffer (pH 6·8; 1 ml.), yeast branching enzyme (45 mg.) and water (4 ml.) was prepared and incubated at 20°. The absorption values (Spekker units) after 0, 0·5, 1·5 and 20·0 hr. were 0·44, 0·21, 0·15 and 0·04 respectively. The digest did not contain reducing sugars (Somogyi reagent and paper chromatography).

Tests for contaminating enzymes. (*a*) Maltase and isomaltase. Yeast branching enzyme (20 mg.) was incubated with maltose, maltotriose and isomaltose (10 mg.) in 0·2 M-sodium citrate buffer (pH 6·8; 0·5 ml.). Paper-chromatographic analysis after incubation at 20° for 24 hr. showed the presence of both glucose and unchanged oligosaccharide. The enzyme preparation thus contains traces of maltase and isomaltase, although the presence of these enzymes will not interfere with measurements of activity of yeast branching enzyme.

(*b*) α-Amylase. The test is based upon that of Hobson, Whelan & Peat (1950) except that glycogen β-dextrin was used. Amylose (5 mg.), amylopectin β-dextrin or glycogen β-dextrin (10 mg.) was incubated with yeast branching enzyme (60 mg.), 0·2 M-sodium citrate buffer (pH 6·8; 2 ml.) and water (8 ml.). Portions (1 ml.) were removed after 0, 20, 60 and 1080 min. The results are shown in Fig. 1. Since the iodine-staining power of glycogen β-dextrin

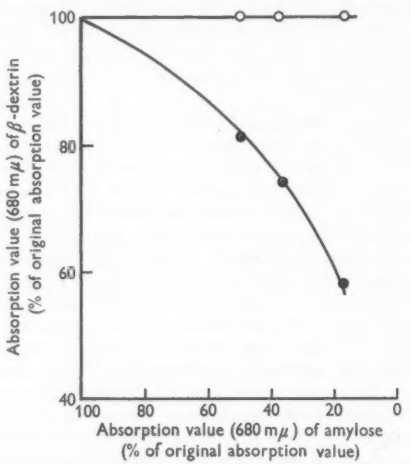

Fig. 1. Detection of α-amylase in preparation of yeast branching enzyme. Relationship between the changes in the absorption value of ○, amylose and glycogen β-dextrin or ●, amylopectin β-dextrin during incubation with yeast branching enzyme.

remained constant it is concluded that yeast branching enzyme is essentially free from α-amylase. The action on amylopectin β-dextrin will be considered later.

(*c*) Isoamylase. Incubation of yeast branching enzyme (1958 preparation) with glycogen gave an insignificant increase in iodine-staining power and β-amylolysis limit. By contrast, preparations made in 1955–56 contained a trace of isoamylase since the β-amylolysis limit of yeast glycogen increased by 4–5% after incubation for 4–14 hr.

(*d*) Phosphorylase. In preliminary experiments the protein precipitated at ethanol concentrations of 17–23 and 23–30% was found to be free from phosphorylase.

RESULTS

Properties of yeast branching enzyme

Effect of enzyme concentration. Although amylose is the normal substrate for branching enzymes, Gilbert & Patrick (1952) have shown that soluble starch may also be used. With this substrate, preliminary experiments showed that with digests containing starch (2%, w/v; 0·5 ml.), 0·2 M-sodium citrate buffer (pH 6·8; 1 ml.), yeast branching enzyme (10–25 mg.) and water to 5 ml., and incubated at 20° for 10 min., activity of yeast branching enzyme as followed by iodine staining was proportional to enzyme concentration. Under these conditions a unit of activity may be defined as the amount of enzyme causing a decrease of 0·1 Spekker absorptiometer unit in the starch–iodine complex. A typical preparation of yeast branching enzyme has an activity of 66 units/g.

Effect of temperature and pH. Figs. 2 and 3 show that action of yeast branching enzyme is optimum at about 20° and pH 7·0.

Effect of possible activators. To determine the effect of maltose, maltotriose, cellobiose and glycogen on the action of yeast branching enzyme on amylose, the following digests were prepared: amylose (2·9 mg.), yeast branching enzyme (15 mg.), 0·1 M-sodium citrate buffer (pH 6·8; 0·5 ml.), activator (5 mg.) and water (3·5 ml.). The reaction was followed by iodine staining. The rate of decrease of absorption value during incubation for 40 min. in a control digest was the same as observed above. The maltosaccharides and cellobiose thus have no effect.

Effect of inhibitors. (*a*) Mercuric chloride. Digests containing amylose (2 mg.), yeast branching enzyme (10 mg. in 0·2 M-sodium citrate buffer, pH 6·8, 0·5 ml.) and mercuric chloride solution (to 2 ml.) were incubated at 20° for 30 min. The results (Table 1) show that yeast branching enzyme

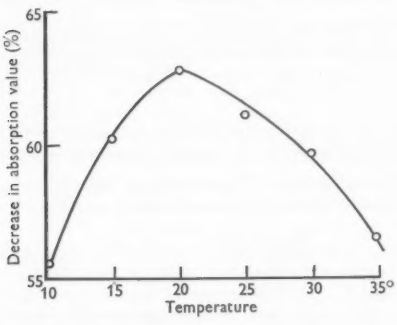

Fig. 2. Effect of temperature on activity of yeast branching enzyme. Amylose (1 mg.) and yeast branching enzyme (20 mg. in 0·1 M-sodium citrate buffer, pH 6·8; 1 ml.) were incubated at various temperatures for 30 min. The reaction was followed by measurements of absorption values with a Spekker absorptiometer.

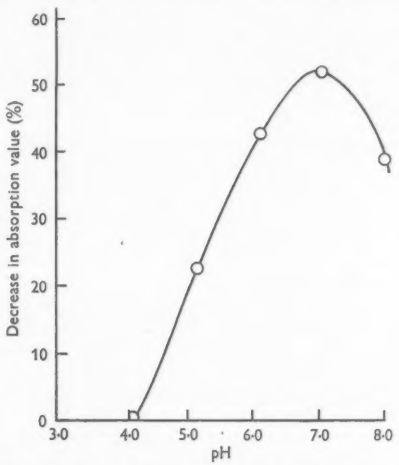

Fig. 3. Effect of pH on activity of yeast branching enzyme. Yeast branching enzyme (100 mg. in 10 ml. of water) was dialysed at 20° against distilled water for 12 hr. Digests contained amylose (2·9 mg.), dialysed yeast branching enzyme solution (1·5 ml.) and 0·2 M-sodium phosphate buffer (1·5 ml.). Absorption values (Spekker absorptiometer readings) were taken after incubation at 20° for 15 min.

Table 1. *Effect of mercuric chloride on the activity of yeast branching enzyme on amylose*

See text for composition of digests. Absorption values are given in Spekker absorptiometer units.

Concn. of mercuric chloride (mM)	Decrease in absorption value	Inhibition (%)
0	0·65	0
0·5	0·00	100
0·2	0·39	40
0·1	0·54	17
0·05	0·60	8

is completely inhibited by 0·5 mM-mercuric chloride.

(*b*) Ammonium molybdate. Amylose (4·5 mg.), 0·2 M-sodium citrate buffer (pH 6·8; 1 ml.), yeast branching enzyme (25 mg.) and ammonium molybdate (12 %, w/v) solution (final vol. 5 ml.) were incubated at 20°. The absorption values of the amylose–iodine complexes were measured at intervals. The results, summarized in Table 2, show that yeast branching enzyme is only partly inhibited by 2 % (w/v) ammonium molybdate.

Action on amylose

Amylose (40 mg.), 0·2 M-sodium citrate buffer (pH 6·8; 5 ml.), yeast branching enzyme (110 mg.) and water (to 25 ml.) were incubated at 20°. After 1, 2 and 4 hr. enzyme action resulted in a 66, 74 and 81 % decrease in absorption value at 680 mμ, and the β-amylolysis limit fell from 84·1 % to 50·4, 49·3 and 48·6 % respectively. These results differentiate yeast branching enzyme from an α-amylase.

The change in absorption spectrum of the amylose–iodine complex during action of yeast branching enzyme is shown in Fig. 4. After prolonged incubation the residual polysaccharide showed maximum absorption at 520 mμ, the solution giving a brown stain. No increase in reducing power was detected.

The above-described changes indicate the formation of branch points in the amylose, the nature of which was examined enzymically. Amylose (32 mg.), 0·2 M-sodium citrate buffer (pH 6·8; 4 ml.) and yeast branching enzyme (80 mg. in 4 ml. of water) were incubated at 20° for 4·5 hr. The enzyme was inactivated by heating (3 min. at 98°) and the digest was cooled and divided into three parts. To part A, water (1 ml.) and β-amylase solution (2 ml.; 800 units) were added; to part B, water (1 ml.) and α-amylase solution (2 ml.) were added. After incubation at 35° for 24 hr., the apparent percentage conversions into maltose were A, 45·4; B, 91·9. To part C, isoamylase solution (25 mg. in 1 ml. of water) was added. After incubation at 20° for 18 hr., the isoamylase was deactivated by heating (3 min. at 98°), cooled and β-amylase solution (2 ml.; 800 units) added. The β-amylolysis limit was 65·3 %. The results show that the branched polysaccharide synthesized from amylose still contains a high proportion of α-1:4-glucosidic linkages, and that many of the newly formed inter-chain linkages are susceptible to isoamylase. Since isoamylase is specific for α-1:6-glucosidic linkages (Manners & Khin Maung, 1955*b*; Gunja, Manners & Khin Maung, in preparation), this characterizes many of the new inter-chain linkages.

The above evidence is consistent with the conversion of amylose by yeast branching enzyme into an amylopectin-type polysaccharide. The properties of the latter were then studied in detail.

Preparation of synthetic amylopectin

Amylose–butanol complex (about 0·5 g.) dissolved in water (250 ml.) was incubated at 20° with yeast branching enzyme (900 mg. in 10 ml. of

Table 2. *Effect of ammonium molybdate on activity of yeast branching enzyme on amylose*

See text for composition of digests. Absorption values are Unicam spectrophotometer readings at 630 mμ.

	Decrease in absorption value (%)		
Time of incubation (hr.)	1·0	2·5	4·5
Control	26	41	58
Ammonium molybdate (1 %, w/v)	21	32	50
Ammonium molybdate (2 %, w/v)	16	22	29

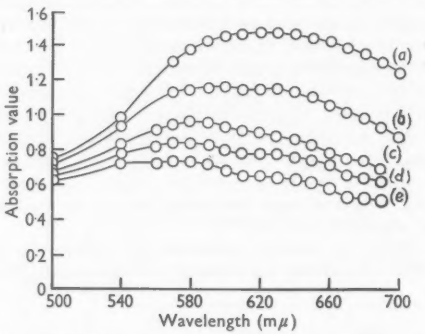

Fig. 4. Light-absorption curves of iodine-stained products during action of yeast branching enzyme on potato amylose. Age of digest (min.): (*a*) 0; (*b*) 10; (*c*) 30; (*d*) 60; (*e*) 120.

Table 3. *Conversion of amylose into an amylopectin-type polysaccharide by yeast branching enzyme*

For composition of digest see text. Absorption values are Unicam spectrophotometer readings at 630 mμ.

Time of incubation (hr.)	Decrease in absorption value (%)	$\lambda_{max.}$ (mμ)	β-Amylolysis limit (%)
0	0	630	97·1
2	20·0	—	78·8
6	29·8	—	73·8
10	38·5	585	70·2
20	61·6	570	60·8
25	63·1	570	59·0
30	67·7	570	58·3
48	77·7	550–555	55·1

Table 4. *Effect of yeast branching enzyme on the absorption value of various amylopectin-type polysaccharides*

Polysaccharide (30–40 mg.), 0·2 M-sodium citrate buffer (pH 6·8; 5 ml.), yeast branching enzyme (20–100 mg.) and water in a final vol. of 25 ml. were incubated at 20°. Portions (0·5 or 1·0 ml.) were stained with iodine, at intervals. A Spekker absorptiometer (filter no. 603) was used for waxy-maize starch and a Unicam SP. 500 spectrophotometer at 540 mμ for malted-barley amylopectin and β-dextrin, and at 555 mμ for synthetic amylopectin, to measure the absorption values.

Time of incubation (hr.)	Absorption value			
	Waxy-maize starch	β-Dextrin	Malted-barley amylopectin	Synthetic amylopectin
0	0·160	0·429	0·295	0·525
2	0·085	—	—	—
3	—	0·392	0·270	0·392
4	0·075	—	—	—
24	0·050	0·375	0·230	0·265
48	—	—	—	0·220

Table 5. *Effect of yeast branching enzyme on the β-amylolysis limit of potato amylopectin*

The digest contained potato amylopectin I (42 mg.), 0·2 M-sodium citrate buffer (pH 6·8; 5 ml.), yeast branching enzyme (70 mg.) and water to 25 ml. Portions (5 ml.) were removed at intervals, heated (98° for 3 min.), cooled and β-amylase solution (3 ml.; 1200 units) was added. After incubation at 35° for 24 hr. the maltose content was determined.

Time of incubation (hr.)	0	1	2	3
β-Amylolysis limit (%)	59·6	53·0	52·2	49·5

water) and 0·2 M-sodium citrate buffer (pH 6·8; 40 ml.). Portions were withdrawn at intervals for measurement of absorption value (0·5 ml.) and β-amylolysis limit (4·5 ml.). The latter solution was heated (3 min. at 98°) to inactivate yeast branching enzyme, cooled and β-amylase solution (500 units in 1 ml. of 0·2 M-sodium acetate buffer, pH 4·6) was added, with water to 50 ml. After incubation at 35° for 24 hr., the maltose content was determined. The results are summarized in Table 3, and indicate the absence of isoamylase from the preparation of yeast branching enzyme.

After 48 hr. the digest was heated (15 min. at 98°), cooled and dialysed against successive changes of distilled water for 48 hr. at room temperature. The polysaccharide–protein mixture was then concentrated *in vacuo* at 35° to one-fifth of the original volume. Since the polysaccharide was only partly soluble, coagulated protein could not be removed by filtration. Deproteinization was therefore effected by the toluene–sodium chloride method (Anderson & Greenwood, 1955). Sodium chloride to 0·1 M-concentration was added and the mixture shaken with freshly distilled toluene (⅓ vol.) for 18 hr. The denatured protein was removed from the toluene–water interface and the aqueous solution collected. The deproteinization was repeated three times. The final aqueous solution was dialysed (24 hr., against distilled water) and the polysaccharide isolated by freeze-drying.

The product had the following properties: glucose content, 81%; α-amylolysis limit, 89%; β-amylolysis limit, 57% (before) and 82% (after) treatment with isoamylase; [η] in M-potassium hydroxide, 55·0; the iodine complex showed $\lambda_{max.}$ 550–555 mμ and absorption value $_{max.}$ 1·30. These properties are typical of amylopectin-type polysaccharides (see Table 9). On incubation with yeast branching enzyme a further decrease in iodine-staining power was observed (Table 4).

Action on amylopectin-type polysaccharides

Iodine-staining measurements. Incubation with amylase-free yeast branching enzyme resulted in a marked decrease in iodine-staining power (Table 4), although the rate was much greater with waxy-maize starch and the synthetic amylopectin, both of which have a lower degree of branching than the malted-barley amylopectin and amylopectin β-dextrin. This behaviour distinguishes yeast branching enzyme from the plant Q-enzymes (Peat, Turvey & Jones, 1959).

β-Amylolysis. Under similar conditions an appreciable (10%) decrease in the β-amylolysis limit of potato amylopectin was observed (Table 5).

In view of these results, the product of action of yeast branching enzyme on potato amylopectin was investigated in detail.

Preparation of synthetic glycogen

Amylopectin (1·3 g.; sample II, prepared by thymol fractionation of potato starch var. Kerr's Pink) was dissolved in water (100 ml.). To the solution after filtration through sintered glass (G. 4) was added 0·2 M-sodium citrate buffer (pH 6·8; 30 ml.) and an aqueous solution of yeast branching enzyme (900 mg.; 20 ml.), and the digest was incubated at 20°. Samples (0·5 or 1·0 ml.) were withdrawn at intervals for determination of absorption

value (Unicam spectrophotometer at 540 mμ) and β-amylolysis limit. The results are shown in Table 6.

After 24 hr. the enzyme was inactivated by heating at 98° for 15 min. Coagulated protein was removed by filtration (G. 3 and G. 4 filters) and the filtrate dialysed against running distilled water at room temperature for 48 hr. The solution was concentrated *in vacuo* and the polysaccharide isolated by freeze-drying.

The product had the following properties: glucose content, 69 %; α-amylolysis limit, 82 %; β-amylolysis limit, 46 %; β-amylolysis limit after pretreatment with isoamylase, 66 %.

The polysaccharide was purified by treatment with ice-cold 4 % trichloroacetic acid. The freeze-dried material was dissolved in water (90 ml.), cooled to 0° and 40 % trichloroacetic acid (10 ml.) was added slowly with stirring. After 18 hr. a small precipitate was removed by centrifuging at 0°, and 2 vol. of cold absolute ethanol was added to the solution. The polysaccharide was dissolved in water, reprecipitated with ethanol, washed with ethanol and dried over P_2O_5. Yield, approx. 0·9 g.

The purified polysaccharide was freely soluble in cold water and the solution gave a red–brown stain with iodine. It had the following properties: $[\alpha]_D + 198°$ (*c*, 0·2 in water); glucose content, 95 %; α-amylolysis limit, 79 %; β-amylolysis limit, 47 %; β-amylolysis limit after pretreatment with isoamylase, 75 %; the iodine complex had $\lambda_{max.}$ 480 mμ and absorption value $_{max.}$ 0·56; [η] in 0·1 M-sodium chloride, 11·7. The combined action of isoamylase and β-amylase gave 90 % conversion into maltose; under similar conditions glycogen and amylopectin gave 95 and 84 %.

The polysaccharide (241 mg.) was oxidized with a saturated solution of potassium metaperiodate (120 ml.) at room temperature. Samples (10 ml.) were analysed for formic acid at intervals; after 10, 12, 15 and 16 days, 0·68, 0·82, 0·86 and 0·86 ml. of 0·01013 N-sodium hydroxide were required for neutralization. The final titre corresponds to an average chain length of 13·5 glucose residues.

The remaining solution of periodate-oxidized glycogen (70 ml.) was filtered and neutralized with ethylene glycol (5 ml.). After dialysis against running tap water at room temperature for 3 days the solution was concentrated (5 ml.). A part (1 ml.) of the solution was hydrolysed with 2 N-sulphuric acid at 100° for 2 hr., neutralized with barium carbonate, concentrated and deionized with Amberlite IR-4B and IR-120 resins. No glucose could be detected by paper chromatography. The remaining solution was treated with potassium borohydride (20 mg.) at room temperature for 3 days. The polyalcohol was precipitated with ethanol, washed with ethanol and then hydrolysed with 2 N-sulphuric acid (2 ml.). The neutralized deionized hydrolysate did not contain glucose (tested by paper chromatography).

Table 6. *Conversion of amylopectin into a glycogen-type polysaccharide by yeast branching enzyme*

See text for composition of digest.

Time of incubation (hr.)	Decrease in absorption value (%)	β-Amylolysis limit (%)
0	0	53·2
2	29·6	52·0
5	39·8	51·5
6·5	47·0	50·5
8	56·0	49·5
18	92·0	47·2

Action on glycogen-type polysaccharides

Yeast branching enzyme had no significant effect on the iodine-staining power or β-amylolysis limit of glycogen or Floridean starch [the latter is an algal polysaccharide closely resembling glycogen in many properties (Fleming *et al.* 1956)], as shown in Table 7. The results also confirm the absence of isoamylase from the preparation of yeast branching enzyme.

DISCUSSION

This investigation shows that extracts of brewer's yeast contain an active branching enzyme (systematic name amylo-1:4 → 1:6-transglucosidase) in addition to the previously reported phosphorylase and isoamylase. This finding has been confirmed

Table 7. *Effect of yeast branching enzyme on the absorption value and β-amylolysis limit of glycogen-type polysaccharides*

For composition of digests see Table 4. (*a*) Absorption value; (*b*) β-amylolysis limit.

Time of incubation (hr.)	Rabbit-muscle glycogen		Horse-muscle glycogen		Floridean starch	
	(*a*) (460 mμ)	(*b*) (%)	(*a*) (480 mμ)	(*b*) (%)	(*a*) (540 mμ)	(*b*) (%)
0	0·105	40	0·119	55	0·227	35
3	0·098	41	—	—	0·230	34
24	0·101	41	0·117	56	0·226	35
48	0·108	—	0·118	—	—	—

independently by Hopkins (1955). In the course of purification of yeast phosphorylase (D. J. Manners & Khin Maung, unpublished work), protein fractions precipitated with ethanol showed the least phosphorylase activity and the greatest activity of yeast branching enzyme. Fractionation of yeast proteins with ethanol–sodium citrate at low temperature, as used by Gilbert & Patrick (1952) for the purification of potato Q-enzyme, was therefore carried out. Although all the fractions showed some activity, that obtained at 25–30% ethanol concentration was most active; the partly purified enzyme was finally isolated by freeze-drying in the presence of sodium citrate buffer, pH 6·8. This method has been applied to the related potato enzymes (Barker, Bourne, Wilkinson & Peat, 1950). The majority of the preparations of yeast branching enzyme were substantially free from phosphorylase and isoamylase but contained traces of maltase and isomaltase, the presence of which does not affect the interaction between yeast branching enzyme and starch-type polysaccharides. The detection of traces of α-amylase is extremely difficult; for example, some preparations of β-amylase contain an α-amylase (Z-enzyme) which appears to attack amylopectin β-dextrin but not glycogen β-dextrin (W. L. Cunningham, D. J. Manners, A. Wright & I. D. Fleming, unpublished work). With this proviso, the results in Fig. 1 and Table 7 suggest that a normal α-amylase is not a significant contaminant of the preparation of yeast branching enzyme. Hopkins (1955) has also concluded that α-amylase is absent from brewery yeasts.

The action of yeast branching enzyme on both amylose and amylopectin results in a decrease in both iodine-staining power and β-amylolysis limit (Tables 3–6) without the concomitant production of reducing groups. The introduction of branch points into the substrate rather than random hydrolysis is therefore indicated. Enzyme action on amylose is optimum at pH 7·0 and 20°, these conditions being similar to those of other branching enzymes (Table 8). Yeast branching enzyme, like potato Q-enzyme, is completely inhibited by mercuric chloride, but only partially by ammonium molybdate and does not require a carbohydrate activator or co-substrate. In this respect, *Polytomella coeca* Q-enzyme, the action of which is markedly stimulated by certain carbohydrates (Barker, Bebbington & Bourne, 1953), would appear to be unique (see also Larner & Uwah, 1956).

Action on amylose

The action of yeast branching enzyme results in a marked change in iodine-staining properties, shown by: (1) a large and rapid decrease in absorption value at all wavelengths, and especially at 680 mμ; (2) a displacement in $\lambda_{max.}$ from 630 to about 520–550 mμ. These changes are accompanied by a 50% decrease in β-amylolysis limit. Since some of the newly formed inter-chain linkages can be hydrolysed by isoamylase, this indicates that they are α-1:6-glucosidic in nature. These results may be compared with the similar action of potato Q-enzyme, and the apparent inability of the animal branching enzymes to attack this polysaccharide (Barker & Bourne, 1953; Cori & Cori, 1943).

Under our experimental conditions amylose is converted into an amylopectin-type polysaccharide. This was difficult to purify, and analysis was confined to iodine-staining and enzymic degradation, since protein impurities interfere with periodate oxidation. Nevertheless, the results obtained (Fig. 5, and Table 9) clearly show the close structural relationship between the synthetic and a natural (potato) amylopectin, particularly with regard to P_M values on α-amylolysis, which are directly related to the degree of branching.

The synthetic amylopectin represents an intermediate in the conversion of amylose into glycogen since on reincubation with yeast branching enzyme further branching takes place. The relative proportion of branching enzyme is therefore a major factor in controlling the degree of branching, in accord with the findings of Barker, Bourne, Peat & Wilkinson (1950).

Action on amylopectin

In contrast with plant Q-enzymes, yeast branching enzyme has a marked action on amylopectin-type polysaccharides. On incubation with waxy-maize starch (or its β-dextrin), potato amylopectin or malted-barley amylopectin, the iodine-staining power or β-amylolysis limit of the substrate or both are appreciably reduced (Tables 4–6). The end-product of action of yeast branching enzyme on potato amylopectin appears to be a glycogen-type polysaccharide since the extent of

Table 8. *Properties of branching enzymes*

Source	Optimum pH	Optimum temperature	Reference
Potato Q-enzyme	7·0	21±1°	Barker, Bourne & Peat (1949)
Broad-bean Q-enzyme	7·25–7·5	20	Hobson, Whelan & Peat (1950)
Green-gram Q-enzyme	7·0	24	Ram & Giri (1952)
Polytomella coeca Q-enzyme	7·3	25–33	Bebbington, Bourne, Stacey & Wilkinson (1952)
Yeast branching enzyme	7·0	20	—

Table 9. *Properties of amylopectin–glycogen-type polysaccharides*

Property	Potato amylopectin	Synthetic amylopectin	Rabbit-liver glycogen	Synthetic glycogen	Brewer's-yeast glycogen
Average chain length (glucose residues)	22·0	—	12·6	13·5	13·2
Proportion of 1:6-linkages (%)	4·5	—	8·0	7·4	7·6
Iodine complex					
$\lambda_{max.}$ (mμ)	545	550–555	460	480	430
Absorption value $_{max.}$	1·30	1·30	0·34	0·56	0·34
α-Amylolysis (P_M value)	89	89	78	79	79
β-Amylolysis limit (%)*					
Before pretreatment	53	57	46	47	44
After pretreatment	80	82	78	75	68
Limiting viscosity number					
In 1 M-potassium hydroxide	205	55	—	—	—
In 0·1 M-sodium chloride	—	—	10	12	7

* 'Before' and 'after' refer to pretreatment with yeast isoamylase.

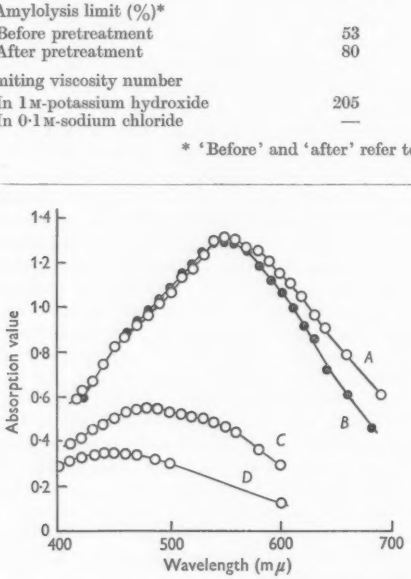

Fig. 5. Light-absorption curves of iodine-stained polysaccharides. Solutions contained 0·01% of polysaccharide (*A*, synthetic amylopectin; *B*, potato amylopectin; *C*, synthetic glycogen) and 0·02% of iodine in 2·0% potassium iodide, and were read against an iodine–iodide reference solution in a Unicam SP. 500 spectrophotometer. The curve for rabbit-liver glycogen (*D*) is taken from Liddle (1956).

enzymic degradation, end-group content and physical properties (especially limiting viscosity number) are very similar to those of brewer's-yeast glycogen and a normal sample of rabbit-liver glycogen (Table 9). It follows that two classes of 'branching enzyme' may be distinguished: (*a*) those that have no action on amylopectin, e.g. potato Q-enzyme (Peat *et al.* 1959); (*b*) those acting on amylopectin, e.g. liver branching enzyme (Larner, 1955). For this reason it is suggested that the term 'Q-enzyme' be confined to the former class of enzymes (cf. Hopkins, 1955).

The present results suggest that the enzymic conversion of amylose into glycogen occurs through an intermediary amylopectin-type polysaccharide, in agreement with generally accepted views, as reviewed by Barker & Bourne (1953). However, Erlander (1958) has proposed that the starch components are produced by the action of *a* debranching enzyme (not yet detected or isolated) on glycogen, and that amylopectin is a partly debranched glycogen. This theoretical reaction is virtually the reverse of that now demonstrated *in vitro*. It is clear that the detailed mechanism of glycogen and amylopectin synthesis *in vivo* requires further investigation, and that the pathways for synthesis and degradation may, in fact, involve separate enzyme systems [cf. Villar-Palsai & Larner (1958) Robbins, Trant & Lipmann (1959)].

On the basis of partial acid-hydrolysis (Wolfrom & Thompson, 1956, 1957) and periodate-oxidation studies (Abdel-Akher, Hamilton, Montgomery & Smith, 1952; Hamilton & Smith, 1956) it has been suggested that both amylopectin and glycogen contain a small proportion of α-1:3-glucosidic linkages. In view of this, the nature of the inter-chain linkages introduced by yeast branching enzyme into amylopectin was examined, as follows: (*a*) periodate-oxidized synthetic glycogen was converted into the corresponding polyalcohol and hydrolysed with acid: glucose could not be detected; (*b*) the outermost inter-chain linkages of the synthetic glycogen are, like those of the parent amylopectin, susceptible to isoamylase, which has no action on α-1:3-glucosidic linkages; (*c*) the combined action of β-amylase and isoamylase causes 90% degradation. In view of the relatively small amount of synthetic glycogen available for analysis (the chemical studies by the workers cited above

required 10–100 g. quantities), our results are considered to provide qualitative evidence against the synthesis of a significant proportion of α-1:3-glucosidic linkages.

Action on other polysaccharides

The results in Table 7 show that yeast branching enzyme has no significant action on the β-amylolysis limit and iodine-staining properties of normal glycogen (muscle glycogen was used since it is more iodophilic than yeast glycogen). A polysaccharide containing 8% of α-1:6-glucosidic linkages probably represents the highest degree of branching obtainable under these conditions *in vitro*. However, the failure of yeast branching enzyme to act on horse-muscle glycogen (average chain length 17) and ability to react slowly with malted-barley amylopectin (average chain length 18) suggests that the specificity of yeast branching enzyme is not entirely controlled by the degree of branching in the substrate; further, 'glycogen' and 'amylopectin' may differ structurally in an as yet unknown way.

The appreciable action of yeast branching enzyme on amylopectin β-dextrin shows that chains carrying 'stubs' of two to three glucose residues can be transferred intact. This provides further evidence for the view that yeast branching enzyme has a rather wider specificity than its plant and animal counterparts.

SUMMARY

1. Extracts of brewer's yeast contain a branching enzyme (amylo-1:4 → 1:6-transglucosidase) which may be isolated by ethanol–sodium citrate fractionation at −5°.

2. Enzyme action on amylose is optimum at pH 7·0 and 20°, and is inhibited by mercuric chloride (0·5 mM).

3. The reaction is characterized by a marked decrease in iodine-staining power and β-amylolysis limit of the substrate without the concomitant production of free reducing groups.

4. Enzyme action on amylose yields initially an amylopectin-type polysaccharide; on incubation with further enzyme, amylopectin is converted into a glycogen-type polysaccharide.

5. The mode of synthesis of glycogen and the properties of related animal and plant branching enzymes are discussed.

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Studies on Vitamin E

1. THE DETERMINATION OF TOCOPHEROLS IN ANIMAL TISSUES

BY E. E. EDWIN, A. T. DIPLOCK, J. BUNYAN AND J. GREEN
Walton Oaks Experimental Station, Vitamins Ltd., Tadworth, Surrey

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In connexion with a programme designed to study the vitamin E status of animals under different nutritional conditions, a rapid and accurate method of determining tocopherols in animal tissues was required. Two problems exist: the extraction, without loss, of the material sensitive to oxidation and its separation from large quantities of other reducing substances before determination.

The extraction problem was of most immediate interest and has received considerable attention from previous workers in the field. Apart from blood and milk, which cause no special extraction difficulties, some kind of comminution or homogenization of the tissue is usually necessary, and a number of different methods have been used by previous workers. Karrer, Jaeger & Keller (1940) chopped organs as finely as possible and extracted them with ethanol–benzene (1:1). Devlin & Matthill (1942) homogenized rat muscle in the Waring Blendor with the foaming solvent mixture (Skellysolve B–ethanol; 8·5:10·0) of Moore & Ely (1941), three separate extractions being used to ensure complete extraction. Hines & Matthill (1943), in the first systematic study of the vitamin E content of animal tissues, used the same procedure. Emmerie & Engel (1943) first introduced the use of boiling ethanol as solvent. Kaunitz & Beaver (1946) adopted a lengthy procedure, emulsifying muscle first with large quantities of acetone in the Waring Blendor and then with several mixtures of acetone and Skellysolve B. Even after this procedure, reducing substances could still be extracted from the tissue. Kringstad & Folkvord (1949), studying cod roes, homogenized the tissue and extracted with ether. Wanntorp & Nordlung (1947) and more recently Bencze (1954) treated the problem by directly saponifying the whole tissue, but recoveries from this procedure are not given. Quaife & Dju (1949), in one of the best studies on vitamin E analysis, criticized both saponification techniques and the use of the Waring Blendor, and they adopted a dry homogenization technique, which they considered essential for the analysis of animal tissues. The tissues were first diced and then frozen in liquid nitrogen, ground in an iron mortar at $-70°$, sieved and finally extracted with hot ethanol vapour. A 24 hr. extraction period was necessary to remove reducing substances completely. Bratzler, Loosli, Krukovsky & Maynard (1950), studying tocopherol levels in the pig, freeze-dried the tissues and then ground the organs in a Wiley mill and the muscle in a Waring Blendor with solid carbon dioxide, finally extracting with ethanol vapour for 24 hr. Swick & Baumann (1952) used a similar technique, but homogenized smaller samples in ethanol before extraction in a Soxhlet apparatus for 18 hr. Draper & Csallany (1958) comminuted samples of rat and rabbit tissue by grinding with acid-washed sand.

The problems connected with the removal of the extensive reducing impurities from the tocopherol extract were attacked by earlier workers in many different ways and have led to extremely variable estimates for the vitamin E contents of animal tissues. The various analytical modifications that

have been used may be consulted in the *Annotated Bibliography of Vitamin E* (1940–57). For example, Feldheim (1956) has listed 40 references to methods for the analysis of vitamin E in human serum and points out that the variation in the literature figures (0·08–2·00 mg./100 ml.) reflects differences in the purification methods (no extraction difficulties occur with serum and almost all workers have used the same ferric chloride–dipyridyl method for the determination of tocopherol). A novel method of analysis was introduced recently by Nair & Magar (1954), who introduced the phosphomolybdic acid reagent, claimed to be fairly specific for tocopherols (and presumably necessitating less preliminary purification of extracts). The same authors (1956) used this reagent to determine vitamin E in blood, which method was also used by Garton, Duncan, Madsen, Shanks & Beattie (1958) in a study of serum tocopherols in the pig. Rosenkrantz (1957) used the phosphomolybdic acid method in a microprocedure for the analysis of vitamin E in human serum and rabbit tissues.

In the present work, a new method of homogenization and extraction has been used. The tissue is frozen by immersion in acetone at $-70°$ and then ground at this temperature with a mixture of sodium sulphate and acetone. There is rapid conversion into a powder, which can be extracted easily with more acetone in a Soxhlet apparatus. Separation of the tocopherols from each other and from reducing impurities follows previously described methods.

METHODS

Homogenization and extraction

The tissue (20 g., or less if this quantity is not available) is cut into 1 cm. pieces and these are sliced through repeatedly on both sides of the tissue with a sharp scalpel. The scored pieces are then plunged into a bath containing 30 ml. of acetone and solid carbon dioxide (freshly cracked to prevent too much water accumulating). After about 10 min. at $-70°$ the frozen pieces are removed with a scoop and broken into smaller fragments with a pestle and mortar. Then 20–40 g. of anhydrous sodium sulphate is added and the mixture ground under enough acetone to give a fairly fluid paste. The acetone from the freezing bath is then added and grinding continued until a fine paste is obtained. The time taken for the whole grinding operation is normally about 10–15 min., but is less for soft tissues, whereas for tough old tissues it may take 25 min. The paste is transferred to a 43 mm. Soxhlet thimble, the excess of acetone is filtered through and the residue extracted for 3 hr. with 150 ml. of fresh acetone. The residue, after extraction, is a free-flowing powder.

General procedure for tocopherol analysis

The extracts were analysed by methods based on the two-dimensional paper-chromatographic procedure (previously used for plant materials) of Green, Marcinkiewicz & Watt (1955), more fully described in the Report of the Vitamin E Panel of the S.A.C. (1959). The following important modifications were made.

Saponification. Saturated potassium hydroxide (1 ml.) was used for the saponification of the total acetone extract, provided that it weighed less than 1 g. If the weight of lipid was greater (as with crude body fat), the quantities of potassium hydroxide for saponification were increased accordingly.

Removal of sterols. This separation, by freezing in methanol at $-12°$, was necessary for all animal tissues.

Floridin-earth chromatography. This was by the method described in Appendix II to the Report of the Vitamin E Panel. The tocopherol was adsorbed from light petroleum and eluted with 25 ml. of benzene. This modification, which is essential for the analysis of animal tissues, removes much paraffin material that would normally be eluted in the benzene fraction and would interfere with paper chromatography.

Paper chromatography. Because the amount of tocopherol in animal tissues is usually very small (see below) it is desirable to chromatograph as large a quantity as possible, but in certain cases this is not always possible as the addition of too much material to the paper may affect the chromatographic separation. Normally, starting with 20 g. of a tissue such as muscle or liver, the eluate from the floridin-earth column is evaporated and dissolved in 1 ml. of benzene. We have successfully chromatographed half this total extract on one paper when dealing with vitamin E-deficient tissues; the other half is always chromatographed in a duplicate paper and may be sprayed with the Emmerie–Engel reagent, for spot-visualization. With tissues containing more adequate amounts of tocopherol, less may be chromatographed. The two-dimensional separation closely follows the procedure of the Report of the Vitamin E Panel, except that, with extracts from animal tissues, the substitution of 80% (v/v) ethanol for 75% ethanol as mobile phase during the second-dimensional (partition) run gives superior results. This is due to the fact that the chief impurity remaining in the tocopherol extracts has an R value in the first (zinc carbonate-adsorption) dimension very close to that of α-tocopherol. This impurity is easily visible under ultraviolet light (especially if the papers have been rendered fluorescent with sodium fluorescein) as a quenching band. It can be extracted from the paper with ethanol, is only slightly reducing and appears to consist largely of ubiquinone ($\lambda_{max.}$ 272 mμ) and certain related substances. The material is quite insoluble in the mobile phase and remains unmoved during partition chromatography. The α-tocopherol, which is nearly always present in a much smaller amount, may become trapped in the impurity if 75% ethanol is used as mobile phase. If 80% ethanol is used, however, the increase in the partition ensures good separation. In certain cases, where α-tocopherol is the only tocopherol of interest, and where exceptionally heavy amounts of ubiquinone are present, 90–95% (v/v) ethanol is even more satisfactory as mobile phase. In this system α-tocopherol has R_F 0·70.

Determination. After location under ultraviolet light the tocopherol spots were cut out and determined as previously described. Since the net extinctions were considerably lower than those usually met with in the analysis of plant extracts, the preparation of the blank was of prime importance. The instructions for its preparation in the Report

of the Vitamin E Panel should be closely followed. The presence and identity of a tocopherol spot was always confirmed by spraying a second paper with the Emmerie–Engel reagent, and, if necessary, by cochromatography with a specimen of the pure tocopherol.

EXPERIMENTAL AND RESULTS

Variation in the homogenization procedure

Several other systems were investigated for their efficiency in homogenization. These included mixtures of sodium chloride and acetone, methanol and light petroleum (b.p. 40–60°), and similar mixtures with sodium sulphate. The efficiency of breakdown was studied on young pig skeletal muscle and was judged on (*a*) ease of grinding, (*b*) adequacy of subsequent extraction, and (*c*) appearance of the residue after extraction. Poor homogenates were obtained with mixtures containing methanol. Mixtures of tissue with sodium sulphate and light petroleum could not be ground, but treatment with sodium chloride–light petroleum gave good results. Acetone was the best solvent investigated and, as indicated in the description of the final method, sodium sulphate–acetone mixtures gave uniformly good results with all tissues.

Study of extraction procedures

In view of the variety of solvent systems used by previous workers, several extraction methods were investigated. In the first series of experiments, young pig skeletal muscle was used. The tissue (20 g.) was removed from the deep-freeze (in which it had been stored for several days), it was homogenized by the sodium sulphate–acetone method and extracted in a Soxhlet apparatus, three times for 1 hr., with acetone, 150 ml. of fresh solvent being used for each extraction. The experiment was repeated with another 20 g. of tissue, which was extracted five times with hot acetone vapour in an apparatus similar to that of Quaife & Dju (1949). Each extract was evaporated, the residue was saponified and total reducing substances were measured. In Table 1 the results are expressed as net extinctions at 520 mμ. In a further experiment, not included in Table 1, 20 g. of tissue was homogenized and then extracted, first with three successive portions of acetone in a Soxhlet apparatus, and then five times, for 1 hr. each time, with hot acetone vapour. The last-named extracts all contained much reducing material, but when the extracts were purified on floridin earth and chromatographed on paper, they contained no tocopherol. In a second series of experiments, 20 g. samples of skeletal muscle from an adult pig were homogenized and extracted for 2 hr. with acetone, ether or ethanol. Extractions by hot vapour and in the Soxhlet apparatus were compared (Table 2). α-Tocopherol was determined in each extract by the chromatographic method.

These two series show that acetone is a good solvent for tocopherol extraction and that, from homogenates prepared from normal tissues, extraction is probably complete in 2 hr. Ethanol appears to be a poor solvent in a Soxhlet-type extractor (probably related to boiling point and heat of evaporation), but is satisfactory in a vapour extractor. The use of increased extraction times and the use of hot solvent vapours, such as have been used by several other workers, appear to produce undesirable non-tocopherol reducing substances from animal tissues.

In a third series of experiments, the efficiency of extraction from tissues high in tocopherol content was studied. A male and a female rat from the stock colony were each given an oral dose of 5 mg. of α-tocopherol acetate every day for 30 days. They were then killed and samples of their skeletal muscle removed. A portion (20 g.) from each rat was homogenized and extracted twice with acetone in a Soxhlet apparatus for an hour each time, and then for a further hour with ethanol vapour. Each fraction was separately analysed for tocopherol by the full chromatographic procedure. The results (Table 3) confirm that extraction, even from these tissues, is substantially complete in 2 hr.

Table 1. *Effect of successive extractions of pig muscle with acetone*

Pig muscle (20 g.) was extracted successively for 1 hr. periods with 150 ml. volumes of acetone.

Method of extraction	No. of extraction	Total reducing substance (E at 520 mμ)
1. Acetone in Soxhlet apparatus	1	3·30
	2	0·45
	3	0·23
	4	Negligible
2. Hot acetone vapour	1	3·23
	2	1·34
	3	0·45
	4	0·73
	5	0·31; incomplete at 5 hr.

Table 2. *Different methods of extraction of α-tocopherol from samples of pig muscle*

Pig-muscle samples (20 g.) were extracted under various conditions for 2 hr. and the α-tocopherol was determined in each extract by paper chromatography.

Method of extraction	α-Tocopherol (μg./g. of tissue)
1. Acetone, Soxhlet apparatus	1·28
2. Acetone vapour	1·20
3. Ether, Soxhlet apparatus	1·18
4. Ethanol, Soxhlet apparatus	0·64
5. Ethanol vapour	1·26

Table 3. *Completeness of tocopherol extraction from rat muscle*

See text for details.

Extract	Tocopherol (μg./g. of muscle)			
	α-Tocopherol		β-+γ-Tocopherol	
	Male rat	Female rat	Male rat	Female rat
1. First acetone (1 hr.)	1126	1430	132	119
2. Second acetone (1 hr.)	137	128	<2	<2
3. Ethanol vapour (1 hr.)	28	17	<2	<2

Table 4. *Recovery of α-tocopherol after boiling in solvents*

See text for details.

Tocopherol added (μg.)	Solvent	Time of heating (hr.)	Antioxidant added	Tocopherol recovery (%)
250	Acetone	1	None	98·5
50	Acetone	1	None	98·6
250	Acetone	1	Ascorbic acid	100·6
1000	Ethanol	6	Pyrogallol	100·0*
1000	Ethanol	6	None	99·5*

* In the light.

Quantitative studies on grinding and extraction

In Table 4 are recorded the results of some experiments in which small quantities of D-α-tocopherol were heated under reflux in acetone (150 ml.) for periods of 1 hr. In one experiment ascorbic acid was added as an antioxidant. Each solution was evaporated, the residue was put through the saponification procedure and tocopherol then determined. Recoveries of tocopherol were quantitative with and without the addition of ascorbic acid. Boumann & Slater (1957), referring to some findings of Eggitt & Ward (1953), have stated that if small quantities of α-tocopherol are heated in ethanol in the presence of artificial light and absence of an antioxidant, up to 80% is destroyed in 6 hr. They found, further, that the addition of 100 mg. of pyrogallol/mg. of tocopherol did not prevent the destruction of nearly 50% of the tocopherol, and darkness and the presence of an antioxidant were both necessary to obtain quantitative recovery. Since these results conflicted with ours and since we were unable to find a statement in Eggitt & Ward's paper corroborating the losses, we repeated the experiments of Boumann & Slater. α-Tocopherol (1 mg.) was dissolved in ethanol (60 ml.), with and without the addition of 100 mg. of pyrogallol, and the solutions were heated under reflux for 6 hr. in daylight and with an additional 100w incandescent lamp 20 cm. from the boiling solutions. As shown in Table 4, quantitative recovery was obtained from both experiments. We suggest that the results of Boumann & Slater were due to a failure to extract their tocopherol effectively with light petroleum. [This danger is pointed out by Lehman (1955) and we have confirmed it on many occasions; only ether is satisfactory, unless the immiscible solvent is added before dilution with water.] The differences they found in experiments in which pyrogallol was either present or not present would probably be caused by additional losses on subsequent saponification without an antioxidant. In our experience, the only normal analytical procedure in which it is necessary to protect tocopherol by an antioxidant is saponification; during extraction the addition of pyrogallol is not only unnecessary but, in fact, deleterious, as paper-chromatographic analysis shows that non-saponifiable reducing impurities are formed by the prolonged heating of such phenols.

Table 5. *Recovery of α-tocopherol added to tissues and subjected to the complete analytical procedure*

Weight of each tissue was 20 g.

Tissue	α-Tocopherol (μg./g.)	Added α-tocopherol (μg.)	Recovery of added tocopherol (%)
Pig muscle	1·28	5	90
Pig muscle	1·28	100	92
Rat liver	8·3	20	98

Three full recovery experiments were carried out. Two 20 g. samples of adult pig muscle and one 20 g. sample of male rat liver (animal from the stock colony) were homogenized with added α-tocopherol in acetone. The samples were analysed by the full chromatographic procedure. Table 5 shows that the method gives recoveries of over 90%.

Comparison with other methods

We have compared the results obtained by the present method on a number of tissues with results obtained on the same samples by using previously described methods. Two samples of liver, one (A) from an adult rat that had been 14 months on a vitamin E-free diet and another (B) from a stock-colony rat, and a sample of adult pig muscle were analysed by the method of Swick & Baumann (1952). The same two livers and also the kidneys from vitamin E-deficient rats (14 months on diet) were analysed by the method of Rosenkrantz (1957). Two samples of cow serum were analysed by the phosphomolybdic acid method of Nair & Magar (1956) and, in addition, by the two methods described by Rosenkrantz (1957). This author apparently omits the cold-saponification step of Nair & Magar and measures vitamin E by a modification of the phosphomolybdic acid method and also by the normal Emmerie–Engel method. Each extract obtained by the various methods was then analysed by two-dimensional paper chromatography, and, finally, each tissue was analysed by the present method. Thus at least three results were obtained from each tissue: (*a*) the apparent tocopherol content found by following the quoted method, (*b*) the true tocopherol in the same extract, determined after paper-chromatographic analysis, and (*c*) the tocopherol content found by the present method.

The results, grouped in Table 6, show that the Swick & Baumann extraction procedure leads to some loss of tocopherol, but the losses are not great. Nevertheless, the determination by this method of tocopherol in tissues from rats fed on a stock diet gave figures that were 300–600% too high, whereas on the liver (A), which actually contained no tocopherol determinable by paper chromatography (thus less than 0·1 μg./g.), the Swick & Baumann method indicated 22·7 μg./g. This experiment illustrates well the extent of non-tocopherol reducing impurities that may still be present after classical purification processes. Rosenkrantz's procedure extracts only about half the tocopherol from the liver: in spite of this, the 'vitamin E' figures obtained by the method are about ten times too high for the normal liver, and the method gives anomalous results for the deficient liver (A) and kidney.

The serum analyses by Rosenkrantz's methods were respectively about twenty times and four times too high. The analytical procedure of Nair & Magar, on the other hand, destroyed over 90% of the existing tocopherols in the serum, but still gave 'vitamin E 'figures about five times too high.

A study of the phosphomolybdic acid method

The reagent introduced by Nair & Magar (1954) gives a yellow–green colour with tocopherols and this is measured at 725 mμ. Contrary to the original findings of Nair & Magar, Rosenkrantz (1957) found that the reagent reacted to a considerable extent with vitamin A, β-carotene and some other substances. Both groups of workers nevertheless

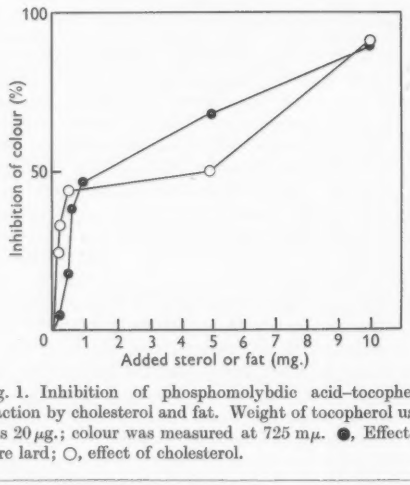

Fig. 1. Inhibition of phosphomolybdic acid–tocopherol reaction by cholesterol and fat. Weight of tocopherol used was 20 μg.; colour was measured at 725 mμ. ●, Effect of pure lard; ○, effect of cholesterol.

Table 6. *Comparison of different analytical methods*

Tocopherols were determined by the following methods: 1, Swick & Baumann (1952); 2, Rosenkrantz (1957), with (*a*) phosphomolybdic acid and (*b*) ferric chloride–dipyridyl; 3, Nair & Magar (1954, 1956); 4*a*, *b* and *c*, paper-chromatographic analysis on methods 1, 2 and 3 respectively; 5, present method.

	Total tocopherols (μg./g.)							
Method	1	2*a*	2*b*	3	4*a*	4*b*	4*c*	5
Tissue								
Pig muscle	7·7	—	—	—	0·81	—	—	1·23
Rat liver A	22·7	120·4	57·2	—	0	0	—	0
Rat liver B	29·8	123·6	—	—	9·3	4·1	—	11·1
Rat kidney	—	43·2	20·8	—	—	0	—	0
Cow serum A	—	48·8	20·1	12·0	—	—	0·21	2·39
Cow serum B	—	37·6	12·2	15·6	—	0·47	0	1·82

used the method for determinations of vitamin E in serum. After obtaining the results described above with the phosphomolybdic acid method we carried out a study of the effect of other substances on the tocopherol–phosphomolybdic acid reaction. Contrary to the findings of Nair & Magar (1954), who stated that the reaction was free from interference from calciferol, carotenoids and cholesterol, it was found (Fig. 1) that small quantities of cholesterol (British Drug Houses Ltd.: Laboratory Reagent) markedly inhibited the reaction. The colour development was also inhibited, to much the same extent, by fat. Contrary, also, to Rosenkrantz we found the reagent unsuitable for the detection of tocopherols on paper chromatograms. Although it is true that the reagent will detect small amounts of tocopherols when these are merely added, as spots, to the surface of ordinary filter paper, the colour reaction is suppressed by all types of impregnated paper (whether of the absorption or partition variety) known to us to be useful for the chromatography of tocopherols.

DISCUSSION

The method described for the homogenization and extraction of animal tissues preparatory to the analysis of vitamin E would appear to have several advantages over previously described methods. Tissue breakdown is exceptionally efficient and simple, and the extraction with hot acetone is rapid and, moreover, gives extracts that are freer from interfering substances than those usually obtained by earlier methods. The procedure has been applied, without difficulty, to many types of tissue and may well prove to be suitable for the extraction of other labile, fat-soluble substances.

The full analytical procedure, from extraction to paper-chromatography separation, gives recoveries of added α-tocopherol of over 90%. Comparison of the results obtained by the method with the results obtained by other methods of analysis were illuminating. The values found by the Swick & Baumann (1952) procedure may be taken as typical of the results of a great number of similar methods, based on the original Emmerie–Engel analysis, and with an activated earth used to remove most of the impurities. Swick & Baumann's careful study is doubly interesting because these authors compared their results with another classical method, that of Quaife & Dju (1949), which includes two unusual methods of purification: hydrogenation over a palladium catalyst and micromolecular distillation. Swick & Baumann showed that their own method, when tested on seven types of rat tissue, gave results consistently lower than did the method of Quaife & Dju (in some cases the latter method gave results several times higher than those of the former). Therefore the present finding that the true tocopherol contents of tissues may be several times lower than the figures found by Swick & Baumann leads to the conclusions that many (and probably all) the literature values for vitamin E contents in animal tissues must be seriously in error.

Studies of the phosphomolybdic acid method of analysis lead to the conclusion that the results that have been obtained by its use, up till now, must be regarded as bearing little relation to real vitamin E figures. The errors consequent on its use may well account, for example, for the results of Garton *et al.* (1958), who found that the addition of tocopherol to the diet of pigs did not apparently increase serum-tocopherol levels. It cannot be stressed too strongly that 'vitamin E' figures obtained without the use of a paper-chromatographic (preferably two-dimensional) separation may be grossly misleading, for non-tocopherol reducing impurities are usually present in amounts much greater than the tocopherol itself, even in the purest extracts obtained by classical methods. Draper & Csallany (1958), for example, have recently found, by a non-chromatographic method, that the 'tocopherol' levels of rabbit muscle and rat liver were apparently not affected by dietary depletion.

SUMMARY

1. A method is given for the analysis of tocopherols in animal tissues. The tissues are first comminuted by grinding with solid carbon dioxide and acetone at −70°, and the material is then extracted with acetone in a Soxhlet apparatus. The resulting extract is analysed for tocopherols by a procedure that includes saponification, removal of sterols, chromatography on floridin earth and two-dimensional paper chromatography.

2. Comparisons of results with those obtained on the same tissues by other methods have shown that paper-chromatographic separation is essential for tocopherol determinations on such tissues, which contain very large amounts of non-tocopherol reducing substances, normally included in the Emmerie–Engel colour determination. It is concluded that many literature figures for vitamin E contents of animal tissues must be seriously in error.

3. The phosphomolybdic acid method has been found unsuitable for the analysis of tocopherols in unpurified extracts of animal tissue, and the dangers involved in its use, particularly for estimations of tocopherols in serum, have been stressed.

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Studies on Vitamin E

2. REVERSAL OF RESPIRATORY DECLINE IN NUTRITIONAL LIVER NECROSIS BY INTRAPORTAL ADMINISTRATION OF TOCOPHEROLS AND OTHER SUBSTANCES

BY J. GREEN, E. E. EDWIN, J. BUNYAN AND A. T. DIPLOCK
Walton Oaks Experimental Station, Vitamins Ltd., Tadworth, Surrey

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The onset of overt symptoms in dietary liver necrosis is preceded by a metabolic lesion that impairs the ability of the liver to maintain its respiratory rate (Chernick, Moe, Rodnan & Schwarz, 1955). By comparing the rates of respiration of liver slices at two different time intervals, the intensity of the defect could be assessed. Chernick *et al.* showed that dietary supplements of vitamin E or factor 3 (naturally occurring selenium compounds) prevented or delayed both the necrotic degeneration and the biochemical lesion. Later, Rodnan, Chernick & Schwarz (1956) described the effects of intraportal administrations of α-, β-, γ- and δ-tocopherol on the metabolic defect and showed that, although these substances did not markedly alter the initial rate of respiration, they arrested the respiratory decline. The four tocopherols showed considerable differences in their activity, α-tocopherol being the most active. It was of interest to extend this study to other tocopherols that have since been isolated and, in view of other work on the connexion between metal ions and vitamin E deficiency (King, Lee & Visscher, 1955; Bunyan, Edwin & Green, 1958; Bunyan, Green, Edwin & Diplock, 1960), to investigate the effect of metal ions.

MATERIALS AND METHODS

Rats. Norwegian hooded rats of both sexes were reared on a stock diet until they were about 45–60 g. in weight. They were fed on the necrogenic diet until the respiratory decline developed, usually in a further 35–55 days, when the rats weighed 60–120 g., depending on their age and weight when started on the necrogenic diet.

Necrogenic diet. This consisted of 45% of sucrose, 10% of glucose, 5% of McCollum's salt mixture, 5% of pure lard (Wilts. United Dairies Ltd.), 30% of dried baker's yeast and 5% of a vitamin B mixture in glucose, with oral doses of vitamins A, D and K to supply respectively 1400 i.u., 100 i.u. and 4 μg./rat/week. The vitamin B mixture supplied to each kg. of diet was: 9 mg. of thiamin, 18 mg. of riboflavin, 90 mg. of nicotinic acid, 9 mg. of pyridoxine, 90 mg. of calcium pantothenate, 2 mg. of folic acid, 0·3 mg. of cobalamin, 90 mg. of inositol, 90 mg. of *p*-aminobenzoic acid and 900 mg. of choline tartrate. The salt mixture included ferric citrate, potassium iodide and potassium alum.

Tocopherols. The 5:7:8-trimethyltocol (α-tocopherol), 5:8-dimethyltocol (β-tocopherol), 7:8-dimethyltocol (γ-tocopherol), 8-methyltocol (δ-tocopherol), 7-methyltocol (η-tocopherol), 5-methyltocol, 5:7-dimethyltocol and tocol were synthetic specimens. The ε-tocopherol was the natural substance, prepared from the unsaponifiable fraction of wheat-bran oil.

Intraportal injections. The procedure of Rodnan *et al.* (1956) was followed, with certain modifications. Rats were

anaesthetized by intraperitoneal injection of sodium pentabarbitone. An incision was made in the abdomen and a small piece of the left lobe of the liver was removed and placed in cold Krebs–Ringer phosphate buffer, pH 7·4 (Umbreit, Burris & Stauffer, 1957). The substance to be tested was dissolved in 0·9% sodium chloride, if it was soluble, or (as was the case with the tocopherols) suspended in Tween 80 emulsion and injected into the portal vein. After 1 min. the needle was withdrawn, bleeding staunched and the incision closed. After 20 min. the rat was killed and the rest of the liver removed and placed in the Krebs–Ringer phosphate buffer.

Assessment of respiratory declines. Three slices were taken from both the pre-injection and post-injection liver samples, the same (left) lobe being used wherever possible and areas free from necrosis were chosen. Slices were prepared with as uniform thickness as possible and, although after the early trials they were not weighed, test slices were generally of the order 80±30 mg. In the earlier phases of the study the slices were weighed and oxygen consumptions were calculated (Table 1). However, since the determination of a substance's activity in reversing the decline in respiration can be made by measurements involving only the rate of fall in oxygen consumption, slices were later used unweighed and the calculations made from the actual volumes of oxygen taken up by each slice. Oxygen uptake was measured by incubation of the slices in Warburg vessels with 3 ml. of oxygenated Krebs–Ringer phosphate buffer containing 0·01 M-glucose. The gas phase was oxygen and, after equilibration for 25 min. at 37·5°, readings were taken at 0·5 hr. intervals for 2 hr. The percentage decline in respiration for each slice was calculated as the ratio of the difference between the volume of oxygen consumed during the first half hour (*a*) and the fourth half hour (*b*) to the volume consumed in the first half hour:

$$(\%\ \text{decline} = [a-b]/a \times 100).$$

The percentage reversal of respiratory decline is given by the ratio of the differences between mean pre-injection and post-injection declines to the mean post-injection decline. This calculation is a reduction of that of Chernick *et al.* (1955), which includes individual slice weights and gives the same results.

Livers that gave initial pre-injection declines of less than 30% were regarded as not having reached the true prenecrotic state, in which the biochemical lesion exists, since declines of up to 30% were occasionally observed even in very young animals on the necrogenic diet. Livers giving initial declines of over 80% were regarded as being beyond the prenecrotic state. Although slices from both these types of liver usually responded to α-tocopherol, only results from livers showing initial declines of between 30 and 80% were included in the data used for the statistical evaluation of potencies.

RESULTS

Tocopherols

In the tables presented, the doses given are uniformly reported as μg./100 g. body wt. of rat and this unit was used for all the subsequent calculations. Table 1 gives some examples of initial oxygen consumptions by liver slices before and after intraportal injections. In agreement with Rodnan *et al.* (1956) no difference was observed whether the injected Tween 80 emulsion contained α-tocopherol or not. Table 2 gives some examples from a series of experiments on α-tocopherol and shows pre- and post-injection declines, together with the percentage reversals of decline.

Table 3 presents the data from which the relative potencies of the nine substances under test were calculated. The potencies relative to α-tocopherol were calculated by plotting percentage reversal against log dose and inspecting for departure from linearity at 0 and 100% response. The best pair of parallel lines to pass through the points from α-tocopherol and each substance in turn was calculated by the method of least squares. Since some doses were given only once and not repeated, error variance was estimated from sums of squares of deviations from the parallel lines. Linearity was

Table 1. *Initial oxygen consumption by liver slices before (a) and after (b) intraportal injections*

Substances injected and amount/100 g. body wt.		Fresh slice wt.* (mg.)	q_{O_2}†	Mean q_{O_2}
Tween 80 (3 mg.)	(*a*)	82·6	260	256
		127·0	184	
		86·7	324	
	(*b*)	87·5	157	278
		57·5	276	
		48·2	402	
Tween 80 (3 mg.) and α-tocopherol (800 μg.)	(*a*)	87·7	228	252
		73·0	282	
		52·1	246	
	(*b*)	98·0	286	267
		70·0	250	
		60·9	264	

* Fresh wt. of slices was obtained from the dry weight of slices after the measurement of oxygen uptake and the moisture content of the liver, determined on a separate sample.

† Oxygen (mm.³) consumed/hr./100 mg. fresh wt. of slice, calculated from the first 30 min. incubation period.

Table 2. *Effect of intraportal injections of α-tocopherol on respiratory decline of liver slices*

The tocopherol was used either in Tween 80 emulsion or in the olive oil–glycerol monostearate mixture of Rodnan, Chernick & Schwarz (1956). When Tween 80 was used the amount was about three to four times the quantity of α-tocopherol in the emulsion.

Tocopherol injected (μg./100 g. body wt.)	Injection vehicle	Mean pre-injection decline (%)	Mean post-injection decline (%)	Reversal of decline (%)
None	Tween*	64·7	61·8	4·6
5	Tween	65·9	61·0	7·5
50	Tween	65·8	40·3	38·8
100	Tween	50·8	9·6	81·0
500	Tween	56·4	8·2	85·5
500	Olive oil–glycerol monostearate	69·2	46·4	33·0

* In many control experiments without tocopherol, amounts of Tween 80 up to 37 mg. were used without any significant effect being observed.

Table 3. *Regression lines for intraportal-potency tests*

y, Reversal (%); *x*, log dose.

Substance	No. of dosages	Range of doses (μg.)	Regression equation	Potency
5:7:8-Trimethyltocol	8	5–625	$y = 45{\cdot}8x - 19{\cdot}6$	100
5:7-Dimethyltocol	4	25–200	$y = 68{\cdot}4x - 70{\cdot}2$	60
5:8-Dimethyltocol	10	11–1780	$y = 33{\cdot}6x - 8{\cdot}0$	55
7:8-Dimethyltocol	10	20–13 800	$y = 15{\cdot}5x - 2{\cdot}0$	5
5-Methyltocol	3	50–200	$y = 41{\cdot}9x - 66{\cdot}1$	7
7-Methyltocol	4	540–1000	$y = 30{\cdot}2x - 82{\cdot}7$	0·4
8-Methyltocol	8	25–2000	$y = 16{\cdot}7x - 18{\cdot}2$	4
Tocol	4	200–1000	$y = 4{\cdot}4x + 6{\cdot}8$	1
ϵ-Tocopherol	4	25–200	$y = 70{\cdot}8x - 96{\cdot}7$	23

thus assumed and could not be tested for statistically. Analysis of variance showed no significant departures from parallelism in the assays of 5:7-dimethyltocol, 5:8-dimethyltocol, 5-methyltocol, 7-methyltocol and ϵ-tocopherol. For these substances, potency estimates and limits were calculated by the method of Fieller (1940) and are given in Table 3. This procedure could not be applied in the assays of 7:8-dimethyltocol, 8-methyltocol and tocol because of significant departures from parallelism. In these assays, potency was estimated from the difference between the mean log dose for the test material and the mean log of α-tocopherol doses giving corresponding responses.

Effect of metal ions

The following metal ions were administered intraportally at doses varying between 5 and 1000 μg. (depending on solubility and toxicity effects): TeO_3^{2-}, $Mo_7O_{24}^{6-}$, Mn^{2+}, Co^{2+}, VO^{2+}, VO_3^-, SeO_3^{2-} and CrO_4^{2-}. The Mn^{2+}, Co^{2+}, SeO_3^{2-} and CrO_4^{2-} ions were also administered in admixture with small quantities of α-tocopherol in order to investigate possible synergistic effects; SeO_3^{2-} ion was, in addition, administered with larger amounts of α-tocopherol in order to observe any antagonistic effect of this ion on the activity of the tocopherol, such as observed by Bunyan *et al.* (1960) in erythrocyte-haemolysis tests. Finally, the Co^{2+} ion was further investigated in admixture with 5:7-dimethyltocol and 5:8-dimethyltocol. Of the ions in the first group, only Co^{2+}, Mn^{2+} and SeO_3^{2-} showed activity in inhibiting the decline of respiration, and these results are given in Table 4. The effects observed with Mn^{2+} and SeO_3^{2-} ions were

Table 4. *Effect of* Co^{2+}, Mn^{2+} *and* SeO_3^{2-} *ions in arresting respiratory decline*

Ion	No. of rats	Dose (μg./100 g. body wt.)	Mean reversal (%)
Co^{2+}	1	10	26
	1	25	36
	3	100	60
	2	200	60
Mn^{2+}	1	5	0
	1	10	0
	1	50	9
	1	100	7
	1	200	13
SeO_3^{2-}	4	10	8
	1	20	10
	1	50	5
	3	600	10
	2	1000	8

slight, even at high doses, but the response appeared definite. The effect of the Co^{2+} ion was reproducible and approximately proportional to the dose administered, and was equivalent to that produced by about half the same weight of α-tocopherol. In a number of trials no synergistic or antagonistic effects of the ions investigated could be clearly demonstrated: if present, they would seem to be small and concealed by the large variation in the test system.

Since selenite is only slightly active intraportally but is able to prevent both hepatic necrosis and the biochemical lesion if incorporated into the necrogenic diet, the effect of adding cobalt and manganese salts to the diet was investigated. Three groups of six rats were used, one group on the necrogenic diet, the other two groups on the diet plus 1 and 10 p.p.m. of Co^{2+} and Mn^{2+} ion respectively (as sulphates). The mean decline in the respiration of slices from livers of rats on the basic necrogenic diet was 41%, compared with 63 and 55% for slices from rats on the diet plus cobalt and manganese respectively. Analysis of variance showed that the increase due to cobalt was significant (P 0·05), but that the increase due to manganese was not significant.

DISCUSSION

In common with Rodnan *et al.* (1956) we have found α-tocopherol to be the most active of the tocopherols in reversing respiratory decline in prenecrotic-liver slices. There are, however, two important differences between the results of these workers and the present results. For α- and β-tocopherol we have obtained significant reversals with much lower doses than was found necessary by Rodnan *et al.* These authors found 190 μg. of DL-α-tocopherol and 533 μg. of D-β-tocopherol necessary for 50% reversal of respiratory decline, whereas from our calculations the amounts needed are approximately 27 and 60 μg. respectively. This may be due to a better dispersion when the substances are injected in Tween 80 emulsion rather than in olive oil–glycerol monostearate emulsion (see, for example, the increased activity found by those workers for the water-soluble 'D-α-tocopherol polyethylene glycol-1000 succinate').

Although our estimated potency for β-tocopherol agrees fairly well with the estimate of Rodnan *et al.* the relative potencies of γ- and δ-tocopherol in the present series differ widely from their figures (83 and 17% respectively of the activity of α-tocopherol). The specimens used by us were synthetic, whereas Rodnan *et al.* used the natural D-isomers, but this fact would appear to be insufficient to account for the differences, since these authors found no difference in the activities of D-α-tocopherol and DL-α-tocopherol. The potencies found by us are in agreement with the usual low activities found for γ- and δ-tocopherol in other biological tests. The high activity of 5:7-dimethyltocol is in agreement with the relatively high biological potency of this substance in other tests (see Bunyan, Green, Mamalis & Marcinkiewicz, 1957; Ward, 1958; Bunyan *et al.* 1960). The low activity of 5-methyltocol is in accord with the activities of other monomethyl tocols in this and other tests *in vivo*.

ε-Tocopherol has been shown by Green, McHale, Marcinkiewicz, Mamalis & Watt (1959) not to be identical with 5-methyltocol, which structure had previously been assigned to it. Recent work in these Laboratories has shown it to be, in fact, 2-farnesyl-2:5:8-trimethylchroman-6-ol, the unsaturated analogue of β-tocopherol (Green, Mamalis, Marcinkiewicz & McHale, 1960). The appreciable activity of ε-tocopherol in arresting respiratory decline parallels the high activity found for it by Bunyan *et al.* (1960) in dialuric acid tests both *in vitro* and *in vivo*.

The metal ions investigated were chosen because of certain relationships with α-tocopherol in vitamin E-deficiency syndromes, such as previously observed by other workers: their activity was particularly interesting in view of the concurrent tests, described by Bunyan *et al.* in the next paper, on their effects in reversing erythrocyte haemolysis. The activity of Co^{2+} ion is outstanding in both sets of experiments whereas Mn^{2+} ion shows some, but much less, activity in the two tests. This 'tocopherol-like' effect of Co^{2+} and Mn^{2+} ions has now been observed in several different and apparently unconnected biochemical manifestations of vitamin-E deficiency. Thus Caputto, McCay & Carpenter (1958) have found that ascorbic acid synthesis is curtailed in the livers of vitamin E-deficient rats, and can be reversed by the addition of Co^{2+} and Mn^{2+} ions to the enzyme preparations (the former ion apparently having synergistic effects also). Corwin & Schwarz (1959) have shown that Mn^{2+} ion has a tocopherol-like effect in restoring succinate oxidation in mitochondria prepared from the livers of vitamin E-deficient rats. In contrast with the effect of Co^{2+} ions in arresting respiratory decline when administered intraportally liver slices from rats fed in diets containing 1 p.p.m. of Co^{2+} ion showed greater respiratory declines than those from similar rats fed on the normal necrogenic diet. (Our results indicate that further work may well demonstrate the same effect with Mn^{2+} ions.) This contrasts with the phenomenon observed with SeO_3^{2-} ion, which, added to rat diets, completely protects against the onset of liver necrosis and prevents the decline in respiration of liver slice (Chernick *et al.* 1955), but,

administered intraportally, has only a marginal effect on respiratory decline.

SUMMARY

1. Eight tocols and one other naturally occuring tocopherol have been compared for their activity in reversing the decline in respiration of liver slices, when administered by injection through the portal vein half an hour before taking the test slices.

2. β-Tocopherol (5:8-dimethyltocol) had 60% and 5:7-dimethyltocol had 55% of the activity of α-tocopherol; but, contrary to results of previous workers, we have found the activity of γ- and δ-tocopherol to be very small. Of the monomethyl tocols 5-methyltocol was the most active. ε-Tocopherol, which is now known not to have the tocol structure, has 23% of the activity of α-tocopherol.

3. The Co^{2+} ion was found to have high activity in this test. Mn^{2+} and SeO_3^{2-} ions had low activity. No antagonistic or synergistic effects could be clearly demonstrated by intraportal administration of mixtures of these ions with tocopherols.

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Studies on Vitamin E

3. THE RELATIVE ACTIVITIES OF TOCOPHEROLS AND SOME OTHER SUBSTANCES *IN VIVO* AND *IN VITRO* AGAINST DIALURIC ACID-INDUCED HAEMOLYSIS OF ERYTHROCYTES

BY J. BUNYAN, J. GREEN, E. E. EDWIN AND A. T. DIPLOCK
Walton Oaks Experimental Station, Vitamins Ltd., Tadworth, Surrey

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Rose & György (1950) discovered the haemolytic action of dialuric acid on erythrocytes from vitamin E-deficient rats and showed that the haemolysis could be prevented by vitamin E in the diet and also by incubating the erythrocytes *in vitro* with α-tocopherol. The same authors (1952) evaluated the relative potencies of α-, β-, γ- and δ-tocopherol in preventing haemolysis both by dosage *in vivo* to vitamin E-deficient rats and by addition *in vitro* to a suspension of erythrocytes from a deficient animal. Friedman, Weiss, Wherry & Kline (1958) have described a bioassay procedure to measure the protective effects of doses of vitamin E *in vivo* and have also reported on the relative potencies of racemic and optically active forms of α- and γ-tocopherol and their esters.

Seven tocopherols have now been described as occurring in Nature. Eggitt & Ward (1953) discovered ε-tocopherol and Eggitt & Norris (1956) offered considerable evidence that the substance was 5-methyltocol. Green, Marcinkiewicz & Watt (1955) found ζ-tocopherol in wheat bran and considered it to be 5:7-dimethyltocol. Green & Marcinkiewicz (1956) found η-tocopherol in rice and identified it as 7-methyltocol. Green, McHale, Marcinkiewicz, Mamalis & Watt (1959) have shown that ε-tocopherol is not 5-methyltocol, and more recent work has shown it to be 2-farnesyl-2:5:8-trimethylchroman-6-ol, the unsaturated analogue of β-tocopherol (Green, Mamalis, Marcinkiewicz & McHale, 1960). The ζ-tocopherol in wheat appears to be the trimethylated member in the same series

of compounds as ϵ-tocopherol and is not identical with 5:7-dimethyltocol.

In the present investigation the activities of tocol, the seven methylated tocols and ϵ- and ζ-tocopherol were compared. In addition, the effects of several antioxidants, detergents and metal ions were studied.

EXPERIMENTAL

Materials. The α-, β-, γ-, δ- and η-tocopherol, 5-methyltocol, 5:7-dimethyltocol and tocol were synthetic specimens. The ϵ- and ζ-tocopherol were natural isomers, isolated from wheat bran.

Vitamin E-deficient diet. Vitamin-free casein (Genatosan Ltd.), 25%; sucrose, 50%; pure Danish lard (Wilts. United Dairies Ltd.), 10%; dried brewer's yeast (Marmite Food Extract Co.), 10%; McCollum's salt mixture, 5%; vitamin A palmitate, 40 i.u./g. of diet; vitamin D_3, 2·4 i.u./g. of diet.

Animals. The animals used were female rats of the Norwegian hooded strain. Sensitivity to dialuric acid-induced haemolysis developed after a few weeks on the diet, but the rats actually used were all 3–12 months old, having been depleted of vitamin E since weaning.

Dialuric acid-haemolysis test. The technique employed was essentially that of György (1951) with slight modifications. Reagents were: iso-osmotic (0·9%) sodium chloride solution, sodium chloride–citrate solution (0·9 g. of sodium chloride and 0·6 g. of sodium citrate/100 ml.), phosphate buffer, pH 7·4 (0·2M-KH_2PO_4, 50 ml., and 0·2M-NaOH, 39·34 ml. made up to 200 ml. with water) and dialuric acid solution (12 mg. of dialuric acid dissolved in 5 ml. of buffer at 60° and diluted with 20 ml. of buffer at room temperature). The dialuric acid solution became inactive after about 30 min. Cleaning of the reaction tubes with detergents and soaps was avoided, only water being used.

Tests in vivo

Collection and testing of blood. The rat's tail was washed in hot water and dried. Blood (0·1 ml.) was collected from a fresh cut at the tip, transferred to 2 ml. of the sodium chloride–citrate solution and mixed gently. The suspension was centrifuged at 2500 rev./min. for 2 min. and the supernatant liquid removed by suction. The cells were resuspended in 2 ml. of 0·9% sodium chloride and again centrifuged and the supernatant removed. Finally, the cells were made into an approximately 5% suspension by gentle mixing with 1 ml. of 0·9% sodium chloride solution. From each suspension 0·25 ml. was pipetted into each of three tubes A, B and C. To the three tubes were added respectively 0·25 ml. of buffer, 0·25 ml. of dialuric acid solution and 8·5 ml. of water. Tubes A and B were kept at 37° for 15 min., at room temperature for a further 45 min. and then the contents were centrifuged. From each supernatant 0·2 ml. was pipetted into 3·3 ml. of water and the extinctions of the resulting solutions (A_1, B_1) and of the control solution (C) were measured on an EEL long-cell absorptiometer, with Ilford filter 605 (maximum transmission at 550 mμ), and with tube A_1 set at zero. The percentage haemolysis was calculated from

$$\frac{\text{Reading } B_1 \times 100}{\text{Reading } C}.$$

Dosage methods in vivo. After preliminary investigation it was decided to use only those rats that showed 80% or more of dialuric acid-induced haemolysis on a preliminary bleeding. The rats were dosed orally by micrometer syringe with olive-oil solutions of the test substances at 4.30 p.m. on one day and bled again at 10.0 a.m. the next day. From each pair of results the 'percentage protection' was calculated:

$$100\left[\frac{\%\text{ haemolysis before dosage} - \%\text{ haemolysis after dosage}}{\%\text{ haemolysis before dosage}}\right].$$

Dosage groups were decided by random selection from suitable rats of approximately the same age, and doses of DL-α-tocopherol acetate were given in parallel with the test doses. Rats could be used again after a rest period of about 14 days.

Haemolysis tests in vitro

Blood was taken from one or more vitamin E-deficient rats and mixed into 5% suspensions as described above. Immediately before each test an emulsion of the test substance was freshly prepared by first mixing it with about four times its weight of Tween 80 and then making up in 0·9% sodium chloride to the required strength. Portions of the suspension of the test substance were mixed with 1 ml. quantities of the blood-cell suspension. Sufficient of a dilute Tween 80 solution in 0·9% sodium chloride was then added to each tube to make up the total amount of Tween 80 to 2 μg., to rule out variations in the amount of Tween 80 caused by taking differing portions of the test substance. Finally, 0·9% sodium chloride was added to produce, in each tube, a total volume of 2 ml. The tubes were held at 37° for 1 hr. and then centrifuged. The supernatant was removed and each batch of 'protected' cells was mixed with 1 ml. of 0·9% sodium chloride to give a suspension to be tested for dialuric acid-induced haemolysis by the method described above for the technique *in vivo*. Tocopherols and the other substances under examination were compared directly with DL-α-tocopherol, each test thus being internally controlled to check such (rare) occurrences as spontaneous haemolysis or inactivity of the dialuric acid solution. Tests in any way unsatisfactory were rejected.

RESULTS

Potencies in vivo

The relative potencies *in vivo* of the compounds tested are set out in Table 1. A twin crossover design was used for the test of 5:8-dimethyltocol, a 14-day interval being allowed between doses, and the potency and limits of error were calculated by the method of Smith, Marks, Fieller & Broom (1944). In fact the crossover showed little advantage over a straightforward dilution assay. The potency of 7:8-dimethyltocol was estimated from a four-point assay evaluated by the method of B.S. 911:1940. With 8-methyltocol, a five-point assay resulted in non-parallel lines and so only an approximate estimate of potency is given. The 5:7-dimethyltocol and 7-methyltocol were tested by

giving a graded series of doses of each in comparison with doses of DL-α-tocopheryl acetate: for the calculation, variance was estimated from the sum of the deviations about the standard and the test lines. Since 5-methyltocol and natural ε- and ζ-tocopherol were only available in small quantities at the time the present work was done, their potencies were assessed by reference to the DL-α-tocopheryl acetate regression line (relating percentage protection to log dose).

The antioxidants *NN'*-diphenyl-*p*-phenylenediamine (DPPD), 2:6-di*tert.*-butyl-4-methylphenol (BHT) and propyl gallate were administered orally in single doses varying between 1 and 50 mg. (the larger doses by stomach tube) and methylene blue was administered in aqueous solution by stomach tube at the same dosage. BHT, propyl gallate and methylene blue were almost inactive at all doses, an oral dose of 50 mg. giving only about 5% protection. However, DPPD was about 3% as active as α-tocopherol, giving complete protection at the 50 mg. dose.

Potencies in vitro. The relative potencies *in vitro* of the tocopherols and tocols are set out in Table 2.

Table 1. *Relative potencies* in vivo *of tocopherols and other compounds against dialuric acid-induced haemolysis*

Substance	Potency	Limits of error (P 0·95)
α-Tocopherol acetate	100	—
5:7-Dimethyltocol	66	29–260
5:8-Dimethyltocol	27	16–49
ζ-Tocopherol (natural)	25	21–42
7:8-Dimethyltocol	20	7–39
5-Methyltocol	3–14	—
ε-Tocopherol (natural)	1–5	—
7-Methyltocol	1	0·5–2·0
8-Methyltocol	0·3	—
Tocol	1–5	—
DPPD	About 3	—
BHT	About 0·5	—
Propyl gallate	About 0·5	—
Methylene blue	About 0·5	—

Table 2. *Relative potencies* in vitro *of tocopherols against dialuric acid-induced haemolysis*

Substance	No. of assays	Potency	Limits of error (P 0·95)
ε-Tocopherol (natural)	4	133	114–156
5:7-Dimethyltocol	4	121 (±19)*	See text
ζ-Tocopherol (natural)	2	106	97–118
5:7:8-Trimethyltocol	—	100	—
7-Methyltocol	8	88	80–101
7:8-Dimethyltocol	2	67	51–86
5-Methyltocol	1	67	57–77
5:8-Dimethyltocol	2	54	39–62
8-Methyltocol	1	26	20–35
Tocol	2	34	26–42

* S.E.

For each test, several doses of the test substance were compared with a series of doses of DL-α-tocopherol. A number of such tests were carried out on each substance, the α-tocopherol usually being in the range 0·05–0·40 μg.

Each separate test was evaluated by the standard formulae for interpreting 'parallel-line assays', i.e. assays in which the response is linearly related to the logarithm of the dose. If X and Y are the mean differences (standard − test) between preparations for the logarithm of the dose and for response respectively, and if B is the pooled estimate for the two preparations of the slope of the log (dose)–response line, then the logarithm of the potency ratio (test to standard) is estimated as

$$L = X - Y/B.$$

Also, if N_1 and N_2 are the numbers of responses obtained to the standard and test preparations, s^2 with n degrees of freedom the estimate of the error variance for a single response, t the 5% level of Student's variate for n degrees of freedom, and s^2/P the estimated variance of B, then (Fieller, 1940) the 95% fiducial (or confidence) limits for the true potency ratio are $L' \pm ts'_L$,

$$L' = X - CY/B,$$

where $$s'^2_L = \frac{Cs^2}{B^2}\left[\frac{1}{N_1} + \frac{1}{N_2} + \frac{C}{P}\left(\frac{Y}{B}\right)^2\right],$$

and $$C = B^2/[B^2 - t^2 s^2/P].$$

(Analysis of variance showed no significant departures from parallelism in any of the tests.)

Successive tests on the same preparation exhibited significant variations in s^2 and B; it was accordingly necessary to use an approximate and somewhat empirical method for combining the several results. Each individual estimate L of the logarithm of the potency ratio was accorded a weight $w_L = 1/s'^2_L$, so that the pooled estimate was $\bar{L} = (\Sigma w_L L)/\Sigma w_L$, and the values of L were checked for concordancy by entering the χ^2 table with

$$\chi^2 = \Sigma w_L L^2 - \bar{L}^2 \Sigma w_L$$

and degrees of freedom one fewer than the number of tests to be combined. The corresponding 'limits of error' were calculated as $\bar{L}' \pm ts'_L$, where

$$\bar{L}' = [\Sigma w_L (X - CY/B)]/\Sigma w_L$$

and $$s'^2_L = 1/\Sigma w_L.$$

The degrees of freedom for t were taken to be the average of those for the individual tests. These formulae are not claimed to be exact (except when the number of assays reduced to one), but would clearly be reasonable, if each assay were so precise that C could be taken as unity and L treated as normally distributed.

Successive assays of 5:7-dimethyltocol were not concordant and so the mean of the four potency

Table 3. *Activity* in vitro *of non-tocopherol compounds against dialuric acid-induced haemolysis*

Substance	Amounts tested (μg.)	Estimated median effective dose (μg.)
α-Tocopherol (for comparison)	0·1–0·5	0·2–0·3
Phytol	5, 10, 50	25
DPPD	0·2, 2	1
BHT	1–20	10
Menaphthone	2–20	10–20
Menaphthone sodium bisulphite	2, 20	>20
Vitamin K_1	0·3–14	2
Vitamin B_{12}	10–200	60–100
Teepol	2, 20, 200	Variable (see text)
Ubiquinone 50	2–160	No activity found
Methylene blue	2, 20	
Vitamin A palmitate	2, 20	
Choline tartrate	2, 10, 50	
Linoleic acid	1, 2, 10	
Cetylpyridinium bromide	2, 10, 20	

Table 4. *Ions showing protection* in vitro *against dialuric acid-induced haemolysis*

Compound	Ion	Range tested (μg.)*	Estimated median effective dose (μg.)
$CoCl_2,6H_2O$	Co^{2+}	1–20	7
$SnCl_2,2H_2O$	Sn^{2+}	2–50	7
$K_2Cr_2O_7$	$Cr_2O_7^{2-}$	4–20	10
K_2CrO_4	CrO_4^{2-}	2–50	33
$MnSO_4,4H_2O$	Mn^{2+}	2–50	40

* The figures in this column and corresponding figures in succeeding Tables refer to the weights of the ions, not of the salts.

estimates is quoted together with the appropriate standard error. Potency estimates for 5-methyltocol and 8-methyltocol were derived from single assays. In this way potencies ranging from 26 to 133% of DL-α-tocopherol were estimated.

In Table 3 the activities of several other substances are summarized. Of the non-ionic compounds tested, only DPPD approached the tocopherols in activity, but some protection was found with BHT, phytol, menaphthone, menaphthone sodium bisulphite, vitamin K_1 and vitamin B_{12} at higher doses. The anionic detergent Teepol was found to have activity in the test but the median active dose varied considerably from batch to batch. One sample showed 50% protection at 20 μg. whereas another showed this activity only at 200 μg. The cationic detergent Cetrimide (cetylpyridinium bromide) was inactive.

Effects of metal ions in vitro. The study of the effects of ions *in vitro* in the haemolysis test arose from a number of observations of the effects of such ions in other systems sensitive to vitamin E deficiency. The ions tested and their various effects are presented in Tables 4–7. They fall into three groups. In the first group (Table 4), three cations, Co^{2+}, Mn^{2+} and Sn^{2+}, and two anions, CrO_4^{2-} and $Cr_2O_7^{2-}$, had full protective activity, the median effective doses ranging from 7 μg. for Co^{2+} and Sn^{2+} ions to 40 μg. for Mn^{2+} ion. Further studies (Table 5) revealed that the protective effects of

Table 5. *Additive effects of ions and tocopherol on dialuric acid-induced haemolysis* in vitro

See footnote to Table 4.

Test no.	Tocopherol or ion used (μg.)	Protection (%) By single substance	Protection (%) By tocopherol + ion
1	Co^{2+} (2·0)	7	—
	α- (0·14)	0	61
	α- (0·20)	14	88
	α- (0·26)	50	97
2	Co^{2+} (2·0)	0	—
	β- (0·2)	0	60
	β- (0·3)	68	92
3	Co^{2+} (2·0)	0	—
	δ- (0·3)	0	11
	δ- (0·4)	0	32
	δ- (0·5)	0	54
	δ- (0·6)	0	78
4	Mn^{2+} (10·0)	0	—
	α- (0·20)	0	83
	α- (0·23)	5	91
	α- (0·25)	0	93
5	Sn^{2+} (4·0)	2	—
	α- (0·24)	4	8
	α- (0·28)	52	76
	α- (0·32)	76	82
6	$Cr_2O_7^{2-}$ (20·0)	45	—
	α- (0·18)	10	66
	α- (0·21)	30	73
	α- (0·24)	39	66
7	CrO_4^{2-} (4·0)	11	—
	α- (0·23)	59	82
	CrO_4^{2-} (8·0)	3	—
	α- (0·23)	59	83

these ions were additive with the protection due to α-tocopherol. The additive action of Co^{2+} ion was also clearly demonstrated with the less-active β- and δ-tocopherol. With Co^{2+} and Mn^{2+} ions these additive effects were so great as to suggest synergism between the ions and tocopherol. However, it is difficult to calculate the magnitude of the purely additive effect because of the large difference between the slopes of the respective log dose–response lines, and so synergism is difficult to prove. Another consequence of this difference between slopes is that, in experiments designed to show the additive effects of the weakly active ions CrO_4^{2-} and $Cr_2O_7^{2-}$, choice of doses is critical. A second group of ions that included Cu^{2+}, Cu_2^{2+}, La^{3+}, Zn^{2+}, SeO_3^{2-}, $AuCl_4^-$ and MnO_4^- exerted a pronounced antagonistic effect on the action of α-tocopherol (Table 6). The Cd^{2+} and Cr^{3+} ions also had a slight antagonistic activity, but noticeably less than the other ions in this group. The antagonistic action of the SeO_3^{2-} ion was powerful enough to oppose even the combined protective effect of both α-tocopherol and cobalt (other antagonist ions were not studied with this combination). Certain substances had a direct haemolytic effect on the erythrocytes during the first incubation in the absence of dialuric acid: these were potassium permanganate, chrome alum, auric chloride, cadmium chloride and sodium tellurite. For the studies on protection and antagonism, these substances were used below their effective haemolytic concentrations. Finally, Table 7 lists the third group of ions, which had no activity when tested.

DISCUSSION

The potencies *in vitro* of tocol and its seven methylated derivatives form groups dependent on the number and position of the substituent methyl groups. The range of potency is not great, tocol itself having about one-third of the activity of the fully methylated α-tocopherol. Methyl groups at

Table 6. *Compounds opposing the protective action of α-tocopherol* in vitro *in the haemolysis test*

See footnote to Table 4.

Compound	Ion (μg.)	Protection (%) α-Tocopherol alone	Protection (%) With ion
Na_2SeO_3	SeO_3^{2-} (16)	11–91	0
Na_2SeO_3	SeO_3^{2-} (3)	3–86 (with 2 μg. of Co^{2+})	5–15
$Zn(CH_3{\cdot}CO_2)_2,2H_2O$	Zn^{2+} (10)	14, 75	6, 3
$KMnO_4$	MnO_4^- (4)	88, 96	0
$HAuCl_4,3H_2O$	$AuCl_4^-$ (3·4)	22–97	0
$CuSO_4,5H_2O$	Cu^{2+} (2)	65, 79	6, 0
Cu_2Cl_2	Cu_2^{2+} (5)	74, 94	9, 3
$La(NO_3)_3,6H_2O$	La^{3+} (10)	77, 90	16, 28
$Cr_2(SO_4)_3,K_2SO_4,24H_2O$	Cr^{3+} (10)	66, 77	53, 64
$CdCl_2$	Cd^{2+} (5)	66, 77, 90	38, 66, 84

Table 7. *Compounds found to be ineffective in the haemolysis test*

See footnote to Table 4.

Compound	Ion	Amounts tested (μg.) Alone	Amounts tested (μg.) With α-tocopherol
$Na_2SeO_4,10H_2O$	SeO_4^{2-}	4–90	18
$H_2TeO_4,2H_2O$	TeO_4^{2-}	3–30	15
Na_2TeO_3	TeO_3^{2-}	3–28	0·1, 0·7
$Ce(NO_3)_3,6H_2O$	Ce^{3+}	2–20	10
$(NH_4)_2Ce(NO_3)_6$	Ce^{4+}	2–20	10
$VOCl_2,2H_2O$	VO^{2+}	3, 13	3
$(NH_4)_6Mo_7O_{24},4H_2O$	$Mo_7O_{24}^{6-}$	16	16
$NiSO_4,7H_2O$	Ni^{2+}	2–50	2
$FeSO_4,7H_2O$	Fe^{2+}	2–10	2, 4
OsO_4	OsO_5^{2-}	14–70	14
$UO_2(NO_3)_2,6H_2O$	UO_2^{2+}	2–50	2
$TlNO_3$	Tl^+	2–50	10
$MgCl_2,6H_2O$	Mg^{2+}	2–20	—
KCl	K^+	2–50	10
K_2SO_4	K^+	2–50	10
$CaCl_2,6H_2O$	Ca^{2+}	2–50	10
$NaNO_2$	NO_2^-	1–30	7
$Na_2S_2O_4$	$S_2O_4^{2-}$	1–37	7

$C_{(5)}$ and $C_{(7)}$ enhance the activity of the tocol molecule, whereas a methyl group at $C_{(8)}$ has a depressing effect. The most active substance in the tocol series is thus 5:7-dimethyltocol, with 121% of the activity of α-tocopherol. Natural ε-tocopherol, which is not a tocol, was found to have an even greater potency and this is in accord with the belief of Green *et al.* (1959) that this substance has a lower molecular weight than the tocols. Voth & Miller (1958) have postulated that the binding of tocopherol to certain proteins such as serum albumin can be accounted for by the formation of hydrogen bonds between the hydroxyl group of the tocopherol and the carboxyl group of the protein. They point out that the three methyl groups in α-tocopherol, by depressing ionization of the hydroxyl, would enhance the possibility of hydrogen-bonding. If the relative potency of the tocols in the dialuric acid test depended solely on their degree of bonding with some protein of the erythrocyte membrane, it would be expected that the potency would be enhanced by methyl groups in any position, since *ortho-*, *meta-* and *para*-methylated phenols are all less ionized than phenol itself. The depressive effect of the $C_{(8)}$ methyl group cannot be accounted for by this explanation, and some other factor must be concerned. Nevertheless, the special effect of the *ortho*-methyl groups in enhancing potency might be related to hydrogen-bonding, since 2:6-xylenol is less ionized than 3:5-xylenol.

Rose & György (1952), working with the D-isomers, estimated the potencies of β-, γ- and δ-tocopherol *in vitro* as respectively 40, 30 and 20% of α-tocopherol. The potencies found by us in the present series (in which the synthetic compounds were used) were respectively 54, 67 and 26%, the activity of γ-tocopherol being considerably higher than was found by Rose & György. The potencies of the substituted tocols *in vivo* are clearly dependent on factors other than structure, probably absorption and transport effects. The most active compound *in vivo* is α-tocopherol. The other tocols form two groups, the dimethyl compounds having intermediate activity and the monomethyl tocols having low activity. Rose & György estimated the potencies *in vivo* of β-, γ- and δ-tocopherol as 20, 4 and 3% respectively of α-tocopherol. These may be compared with the potencies found here, which are, respectively, 27, 20 and 0·3%. The figure for γ-tocopherol is again much higher than was found by Rose & György but is in good agreement with the figure of 21·7% found in a series of tests by Friedman *et al.* (1958). Of four antioxidants tested for their protective effects *in vivo*, only DPPD had appreciable activity. This is in agreement with the findings of Gitler, Sunde & Boumann (1958). These authors and also Friedmann *et al.* (1958) found no protection *in vivo* with BHT and methylene blue, but their dosage regimen was different from ours.

In any discussion of the mechanism of the haemolytic effect and its inhibition, all the foregoing factors must be considered. The most widely held theory of the mechanism of dialuric acid-induced haemolysis (Rose & György, 1950, 1952) relates it to the presence of a peroxidic intermediate formed during the reversible oxidation–reduction of dialuric acid and alloxan. In accordance with this view, tocopherols and other antioxidants protect by removing such an active intermediate. Nitowsky & Tildon (1956) concluded that α-tocopherol might function by inhibiting the oxidase action of haemoglobin on the unsaturated fatty acids of the cell membrane (previously demonstrated by Collier & McRae, 1955). These workers studied hydrogen peroxide-induced haemolysis and reported an inverse relationship between catalase activity and percentage haemolysis in erythrocytes. They found menaphthone almost as active as α-tocopherol in their tests *in vitro*, whereas in the present work there is a big difference in the activity of the two substances. This supports the suggestion of Rose & György (1952) that hydrogen peroxide is not the haemolysing agent in the dialuric acid test.

The effects of the ions tested are difficult to correlate; it is not even certain that all the ions that have activity exert their effect by the same mechanism. The Co^{2+} and Mn^{2+} ions are of special interest since they not only have considerable activity in the test *in vitro* but have pronounced additive and possibly synergistic activity in combination with α-tocopherol. In the preceding paper, Green, Edwin, Bunyan & Diplock (1960) have described the effects of Co^{2+} ions in reversing the respiratory decline in liver slices from rats on a necrogenic diet, and it is tempting to postulate a connexion between the two phenomena. Corwin & Schwarz (1959) have established a relationship between the biochemical lesion in necrotic livers (deficient both in vitamin E and selenium) and the regulation of succinate oxidation in liver mitochondria. They have, in fact, found Mn^{2+} ions to have a vitamin E-like effect in restoring succinate oxidation in mitochondria prepared from the livers of vitamin E-deficient rats. Another manifestation of vitamin E deficiency was studied by Caputto, McCay & Carpenter (1958), who found that enzymic synthesis of ascorbic acid in the livers of vitamin E-deficient rats and rabbits was severely curtailed and that the effect could be reversed by adding Co^{2+} and Mn^{2+} ions to the enzyme preparations. They also observed a large synergistic effect when Co^{2+} ions were added to the enzyme preparations from vitamin E-containing livers but found no such effect with Mn^{2+} ions; this somewhat parallels our findings on the relative activity of Co^{2+} and

Mn^{2+} ions in reversing dialuric acid haemolysis. Caputto *et al.* also commented on the slight inhibitory effects of Zn^{2+} and Cd^{2+} ions on ascorbic acid synthesis.

It does not seem possible to correlate the various effects of the ions with their charges, although the activity of Teepol indicates that charge alone may play some part. It appeared important to try to discover whether haemolysis was produced by a surface effect on the erythrocyte membrane (in which ionic charge might be expected to be influential) or whether haemolysis was the culmination of a more deep-seated interference with the metabolism of the cell. Some measurements of erythrocyte mobility showed that, compared with normal cells, those from vitamin E-deficient rats had a slightly higher negative charge, with a greater population variance, and this variance was noticeably reduced when the cells were incubated with α-tocopherol. (This variability of charge from cell to cell may help to explain how it is possible to get, say, 50% haemolysis and not an all-or-none response.)

Two metal-containing anions, CrO_4^{2-} and $Cr_2O_7^{2-}$, and the Sn^{2+} cation were as effective as Co^{2+} and Mn^{2+} ions in inhibiting haemolysis. The Sn^{2+} ion is difficult to keep in solution and part of the effect of this ion may have been due to deposition of an insoluble salt on the surface of the cells. It is difficult to fit the action of the powerfully oxidizing CrO_4^{2-} and $Cr_2O_7^{2-}$ anions into the same picture as the Co^{2+} and Mn^{2+} cations. The only enzymic connexion of CrO_4^{2-} ion, as far as we are aware, is its catalysis of the hydration of carbon dioxide to carbonic acid, which action it shares with carbonic anhydrase and the ions of several other oxy-acids (Roughton & Booth, 1938).

The group of substances that oppose the protective action of α-tocopherol *in vitro* includes both cations and anions, and again it is difficult to correlate their activities with any particular chemical or electrical properties. The group includes the reducing anion SeO_3^{2-} and the oxidizing anion MnO_4^-, the reducing cation Cu_2^{2+} and the oxidizing cation Cu^{2+}. In contrast with the effect of SeO_3^{2-}, the SeO_4^{2-} ion is without activity. The antagonistic action of selenite is of particular interest in view of the vitamin E-like activity of selenite in protecting against dietary liver necrosis in rats (Schwarz & Foltz, 1957), the exudative diathesis in chicks (Dam, Nielsen, Prange & Søndergaard, 1957) and white-muscle disease in lambs (Muth, Oldfield, Remmert & Schubert, 1958). In contrast, Green, Edwin *et al.* (1960) found that intraportal selenite has practically no effect in arresting respiratory decline of necrotic-liver slices. Roughton & Booth (1938) find selenite to be more active than chromate in catalysing the hydration of carbon dioxide to carbonic acid.

The haemolysis of vitamin E-deficient erythrocytes may be a more useful system for investigating some of the metabolic functions of α-tocopherol than has hitherto been recognized. It is possible that the role of vitamin E in preventing haemolysis may be related to enzymic or other type of catalytic process in the cell and that a simple theory of combination with peroxide may not be sufficient to account for the phenomenon. It is perhaps of some interest that Vincent & Blackburn (1958), in a study of the effect of several metal ions on normal human erythrocytes, divided the ions into two groups: those that caused a loss of K^+ ion from the cells, which included Au^{3+}, Cu^{2+}, Cd^{2+} and Zn^{2+} ions, and those that did not cause a loss of K^+ ion, which included Mn^{2+} and Co^{2+} ions.

SUMMARY

1. The potencies *in vitro* and *in vivo* of eight tocols and two other naturally occurring tocopherols in preventing dialuric acid-induced haemolysis in rat erythrocytes have been studied, and some observations made on the relationship between structure and activity. The potencies *in vitro* cannot be clearly related to structure, although it appears that the presence of methyl groups *ortho* to the hydroxyl group in the tocol structure enhances activity somewhat. Hydrogen-bonding, as postulated by Voth & Miller (1958), would not seem to account sufficiently for the differences in potency. *In vivo*, the dimethyl compounds all have considerably greater activity than the monomethyltocols.

2. The activities of a number of other substances in the test have been recorded.

3. The Co^{2+}, Mn^{2+}, Sn^{2+}, CrO_4^{2-} and $Cr_2O_7^{2-}$ ions have been found to be active in preventing haemolysis *in vitro* and the action of these ions is additive to that of α-tocopherol. With Co^{2+} and Mn^{2+} ions the combined effect is so great as to suggest synergism.

4. The Zn^{2+}, Cu^{2+}, Cu_2^{2+}, La^{3+}, Cr^{3+}, Cd^{2+}, MnO_4^{2-}, $AuCl_4^-$ and SeO_3^{2-} ions antagonize the action of α-tocopherol.

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Interrelationships between Folic Acid and Cobalamin in the Synthesis of Methionine by Extracts of *Escherichia coli*

BY R. L. KISLIUK*† AND D. D. WOODS
Microbiology Unit, Department of Biochemistry, University of Oxford

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Gibson & Woods (1960) showed that washed suspensions of various strains of *Escherichia coli* synthesize methionine from homocysteine; serine was an effective source of the methyl group. With *p*-aminobenzoic acid and cobalamin auxotrophs they also showed these vitamins to be essential for the reaction. Parallel inhibition by sulphathiazole of both the synthesis of methionine from homocysteine and of folic acid from *p*-aminobenzoic acid gave strong, though indirect, evidence that *p*-aminobenzoic acid must first be converted into some form of folic acid before it exerts its function. However, no folic acid derivative tested was able to replace *p*-aminobenzoic acid for methionine synthesis by the intact organisms.

Enzymic extracts of an auxotroph of *E. coli* requiring serine or glycine for growth were obtained by Cross & Woods (1954) by grinding with alumina and by Szulmajster & Woods (1955, 1960) by extracting acetone-dried organisms. Both preparations synthesized methionine from homocysteine and serine and there was, among other factors, a partial requirement for an extract of heated *E. coli*. The last-named authors partly purified the heated extract and obtained evidence that it contained a folic acid derivative. With neither source of enzyme could the heated extract be replaced by either tetrahydropteroylglutamic acid or its N^5-formyl derivative (leucovorin). This was surprising, because although tetrahydropteroylglutamic acid may not be the natural folic acid coenzyme (Wright, 1958) it is effective in replacing it in all other C_1-transfer reactions so far studied.

The object of the present work was to investigate this point further and to try to find conditions under which tetrahydropteroylglutamic acid could be used. Since cobalamin is known to be required for methionine synthesis in *E. coli* by growing cultures (Davis & Mingioli, 1950), washed suspensions (Gibson & Woods, 1952, 1960) and enzymic extracts (Helleiner & Woods, 1956) of cobalamin auxotrophs, special attention was paid to the effect of this vitamin. It was found that growth in the presence of added cobalamin permitted the utilization of tetrahydropteroylglutamic acid by enzymic extracts of acetone-dried organisms. Brief reports of some of the results have been given (Kisliuk & Woods, 1957, 1959).

* Fellow of the National Foundation for Infantile Paralysis, U.S.A.
† Present address: National Institutes of Health, Bethesda 14, Maryland, U.S.A.

MATERIALS AND METHODS

Organism. Escherichia coli PA 15, an auxotrophic strain requiring serine or glycine for growth, was used throughout. It was obtained originally from Dr B. E. Wright and was maintained as described by Gibson & Woods (1960). The strain was chosen because when grown on glycine it synthesizes methionine more actively than other strains tested (Gibson & Woods, 1960); it was also used in these Laboratories by Cross & Woods (1954) and Szulmajster & Woods (1955, 1960) for the preparation of enzymic extracts which synthesize methionine.

Medium and growth. The glucose–inorganic salts medium of Davis & Mingioli (1950), supplemented with glycine (0·8 g./l.), was used except that the glucose was autoclaved with the other constituents. The organisms were grown semi-anaerobically in 5 l. culture volume in 5 l. flasks plugged with cotton wool for 48 hr. at 37°. The inoculum was 5 ml. of 18 hr. culture grown in the same medium. The yield of organisms was about 0·2 g. dry wt./l.; this was increased by about 20–25% when cobalamin (4 μg./l.) was added to the growth medium. The organisms were harvested in a continuous centrifuge and washed once with one-twentieth of the culture volume of water.

Preparation of enzymic extract. Organisms from 25 l. of medium were added to 2·5 l. of acetone at −15°, shaken vigorously and set aside at −15° for 20 min. The precipitate was collected on a sintered-glass funnel, washed three times with 100 ml. quantities of acetone at −15° and placed in a vacuum desiccator, which was exhausted for 3 hr. with a water pump. The powder (yield 0·16 g./l. of medium without cobalamin) retained full activity for at least 6 months when stored in a sealed container at −15°.

Acetone-dried organisms (1 g.) were extracted with 14 ml. of water by rubbing with a glass rod for 20 min. The extract was centrifuged (25 000 *g*; 45 min.) at 0° and the supernatant dialysed for 14 hr. at 3° against 0·05 M-phosphate buffer, pH 7·8; it then contained 7–10 mg. of protein/ml. The ratio of absorption at 280 mμ to that at 260 mμ was 0·62–0·7, indicating a nucleic acid content of 10–17%.

From some experiments the dialysed extract was treated with Dowex-1 resin to remove residual folic acid coenzyme. The extract (10 ml.; 7·5 mg. of protein/ml.; 280 mμ/260 mμ ratio 0·62) was passed through a short column (1·5 cm. × 3 cm.) of Dowex-1 (chloride form, ×2, 50–100 mesh) at 3°; the column was washed with water until the original volume of solution was obtained (6·8 mg. of protein/ml.; 280 mμ/260 mμ ratio 0·7).

Bulk production of enzymic extracts. In the latter part of the work it was necessary to have a uniform source of enzymic extract for assaying the factor formed in the presence of cobalamin which permits the utilization of tetrahydropteroylglutamic acid. A large batch of organisms was grown on the surface of agar medium in trays and the product acetone-dried as described by Szulmajster & Woods (1960). We are greatly indebted to the Director and staff (particularly Mr F. C. Belton) of the Microbiological Research Establishment, Ministry of Supply, Porton Down, Wilts, for undertaking the growth of the organisms. The yield of organisms was higher than with the semi-anaerobic liquid cultures normally used but the activity of the enzymic extracts was 20–30% less.

Extract of heated Escherichia coli. Acetone-dried organisms (0·5 g.) were rubbed up with 3 ml. of water and heated at 100° for 3 min. The supernatant after centrifuging was stored at −15°.

Test for methionine synthesis. The standard reaction mixture used to test methionine synthesis by enzymic extracts was based on the work of Cross & Woods (1954) and Szulmajster & Woods (1960). It contained, in 2 ml. final volume of potassium phosphate buffer, pH 7·8 (containing 200 μmoles of phosphate ion): DL-homocysteine (20 μmoles); L-serine (10 μmoles); hexose diphosphate (15 μmoles); adenosine triphosphate (ATP; 10 μmoles); diphosphopyridine nucleotide (DPN; 1 μmole); pyridoxal phosphate (1 μmole); $MgSO_4$ (10 μmoles); the final pH was 7·4. Any changes from the standard mixture are noted for individual experiments; in particular the DL-homocysteine concentration was usually decreased when the formaldehyde derivative of tetrahydropteroylglutamic acid replaced serine as donor of C_1 units.

The reaction was carried out in a final volume of 2 ml. in Thunberg tubes filled with H_2 or N_2, which were incubated at 37°. The reaction was stopped by placing the tubes in a boiling-water bath for 3 min.; after centrifuging off the precipitate the supernatant fluid was assayed for methionine and other products.

Estimation and detection of methionine. The microbiological assay with *Leuconostoc mesenteroides* P 60 described by Gibson & Woods (1960) was followed exactly. Standards included in each assay contained 20, 40, 80, 120 and 160 μm-moles of DL-methionine; each experimental sample was assayed in quadruplicate. The organism responds only to L-methionine. Apart from the specificity tests noted by Gibson & Woods (1960) it did not respond to cobalamin (2 μmg./ml.), DL-1:3-thiazane-4-carboxylic acid (Wriston & Mackenzie, 1957), L-cystathionine, DL-methionine methylsulphonium iodide (all at 50 μm-moles/ml.) or to the initial constituents of the standard reaction mixture; the latter mixture also did not inhibit the response to L-methionine.

In some experiments the production of methionine was verified by chromatography on Whatman no. 1 paper. The chromatograms were developed with butanol–formic acid–water (77:10:13, by vol.) (Strack, Friedel & Hambsch, 1956), which separates methionine (R_F 0·57) from 1:3-thiazane-4-carboxylic acid (R_F 0·40); the latter is formed spontaneously from homocysteine and formaldehyde at neutral pH. Typical reaction products in which methionine was indicated by microbiological assay showed a ninhydrin-positive spot with the same R_F as authentic L-methionine: furthermore, this was the only region of the chromatogram which satisfied the growth requirement of *L. mesenteroides* P 60.

Estimation of serine. The method used was modified from that of Frisell, Meech & Mackenzie (1954). The reaction was stopped by adding 15% (w/v) trichloroacetic acid (0·8 ml.) and the precipitate removed by centrifuging. The supernatant (1·5 ml.) was pipetted onto a column of Dowex-50 resin (H^+ form, ×8, 100–200 mesh, 0·5 cm. × 4 cm.) which was then washed with water (20 ml.) to remove any free formaldehyde. Serine was eluted with 4 N-HCl (20 ml.), collected in 25 ml. beakers and evaporated to dryness on a hot plate. The residue was taken up to 1 ml. with water plus a drop of methyl red (made up in a dilute acid), neutralized with 0·25 N-NaOH and again evaporated to dryness.

The residue was thoroughly mixed with 0·075 M-sodium metaperiodate (1·5 ml.) and set aside for 15 min. The solution was poured into a short column (1 cm. × 3 cm.) of Dowex-1 resin (chloride form, ×2, 50–100 mesh); this retains periodate but allows formaldehyde formed from the β-C of serine to pass through. A total volume of 10 ml. of eluate and washings was collected. Formaldehyde was assayed on appropriate samples with the acetylacetone reagent of Nash (1953).

When DL-homocysteine was present in the reaction mixture it was necessary to separate serine from 1:3-thiazane-4-carboxylic acid, which is also broken down by periodate to yield formaldehyde. This was achieved by substituting a column of Amberlite IR 120 resin (60–100 mesh, 0·8 cm. × 18 cm.) for the Dowex-50 used in the first treatment above. After washing the column with water the serine was eluted with N-HCl (15–40 ml.) and the estimation continued as described above. The thiazane-carboxylic acid (which is always present in the products) may arise by reaction of homocysteine either with the formaldehyde derivative of tetrahydropteroylglutamic acid or with free formaldehyde formed from the β-C of serine.

Serine formation was verified in typical experiments by chromatography on Whatman no. 1 paper. The developing agent, which separates serine (R_F 0·71) from glycine (R_F 0·56), was triethylamine–acetone–water (5:80:15, by vol.) (Korkes, S. cited by Wright & Stadtman, 1956).

Assay of cobalamin. The method used was modified from that of Hutner *et al.* (1949); the assay organism was *Euglena gracilis* var. *bacillaris*. Medium S of Lascelles (1956) was supplemented with (per l. final volume): (*a*) acid-hydrolysed casein (vitamin-free, prepared by the method of Snell & Rannefeld, 1945) equivalent to 0·5 g. of casein and (*b*) 1·3 ml. of 4 mM-iron citrate (prepared as described by Lascelles, 1956). The pH was adjusted to 4·5 instead of 6·8.

The medium (5 ml. final volume in 16 mm. × 125 mm. tubes) was autoclaved (10 min. at 115°) after the addition of cobalamin or experimental samples. Standards covering the range 0–6 μμg. of cobalamin/ml. were included in each assay. The inoculum was derived from a 7-day culture on the same medium containing 4 μμg. of cobalamin/ml.; the organisms were centrifuged out, resuspended in the culture volume of 0·9% NaCl and 0·05 ml. of the suspension was added to each assay tube. Incubation was for 10 days at 30° and 1 ft. distant from two 60 w tungsten-filament lamps. Growth was assessed in an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with a neutral-density filter.

Estimation of protein. This was determined spectrophotometrically according to Layne (1957). The ratio of absorption at 280 mμ and at 260 mμ also gave information on the relative amount of nucleic acid present.

Tetrahydropteroylglutamic acid. Pteroylglutamic acid (folic acid) was catalytically reduced by the method of O'Dell, Vandenbelt, Bloom & Pfiffner (1947). The product was assayed by iodine titration or by treatment with excess of formaldehyde and estimating the unbound formaldehyde with Nash (1953) reagent; 70% was capable of binding formaldehyde and was presumably tetrahydropteroylglutamic acid.

The formaldehyde derivative of tetrahydropteroylglutamic acid (tetrahydropteroylglutamic acid–formaldehyde) was prepared (in solution) by mixing equimolar solutions (usually 0·035 M) of the acid and formaldehyde in M-phosphate buffer, pH 7·8, under an atmosphere of H_2 (Kisliuk, 1957).

Chemicals. The ATP, DPN and triphosphopyridine nucleotide (TPN) were products of the Sigma Chemical Co., St Louis, Mo., U.S.A. Hexose diphosphate (barium salt) and cobalamin (Anacobin) were from British Drug Houses Ltd., London. Pyridoxal phosphate (Roche Products Ltd., Welwyn Garden City, Herts), protamine sulphate (from herring roe) (L. Light and Co. Ltd., Colnbrook, Bucks) and DL-homocysteine (Nutritional Biochemicals Inc., Cleveland, Ohio, U.S.A.) were also commercial specimens.

RESULTS

It should be emphasized that positive results with cobalamin were obtained only when it was added to the medium on which the organisms were grown; it had no effect when added to the enzymic extracts of acetone-dried organisms harvested from a medium to which cobalamin had not been added.

Inhibition of methionine synthesis by tetrahydropteroylglutamic acid

With dialysed (though not Dowex-1-treated) enzymic extracts of organisms grown without cobalamin substantial synthesis of methionine occurs with L-serine as C_1 donor; there is presumably sufficient coenzyme form of folic acid remaining after dialysis to catalyse the transfer. This synthesis is, however, abolished if tetrahydropteroylglutamic acid is added (Fig. 1, no cobalamin in medium): 0·3 μmole in 2 ml. of test solution completely inhibits and 50% reduction is given by 0·1 μmole. The inhibition is not overcome by adding to the reaction mixture a heated extract of *E. coli* (presumed to contain the natural folic acid coenzyme) or cobalamin (37 μm-moles). The mechanism of this inhibition is unknown and has not been further explored.

Influence of growth in the presence of cobalamin

Serine as one-carbon-atom donor. Methionine synthesis did occur from serine and homocysteine in the presence of tetrahydropteroylglutamic acid provided that the organisms were grown in a medium containing cobalamin; increasing concentration of cobalamin (up to about 1 μmg./l. of medium) yielded extracts of increasing activity (Fig. 1). By contrast methionine synthesis in the absence of tetrahydropteroylglutamic acid (natural folic acid as coenzyme) was markedly diminished by low concentrations of cobalamin in the medium; at higher concentrations it was partially restored (Fig. 1). These results possibly suggest that a new pathway for methionine synthesis results from growth on cobalamin, a pathway in which tetrahydropteroylglutamic acid is used in place of the natural coenzyme.

Formaldehyde derivative of tetrahydropteroylglutamic acid as one-carbon-atom donor

Free formaldehyde will not serve as source of the methyl group of methionine with either intact organisms or enzymic extracts of *E. coli* PA15. It is probable that it is inactivated by spontaneous reaction with homocysteine to form 1:3-thiazane-4-carboxylic acid (Wriston & Mackenzie, 1957), which is biologically inert. Tetrahydropteroylglutamic acid–formaldehyde (Kisliuk, 1957) is also inactive with enzymic extracts from organisms grown without cobalamin. Growth with cobalamin, however, confers activity in this respect also (Fig. 1). The results of Fig. 1 give a falsely low impression of the activity of tetrahydropteroylglutamic acid–formaldehyde; the concentration of homocysteine used (10 μmoles/ml.) was optimum for serine as donor whereas the optimum concentration for tetrahydropteroylglutamic acid–formaldehyde is only one-third of this and shows a sharp maximum (Fig. 2). It is possible that the tetrahydropteroylglutamic acid–formaldehyde is decomposed by the higher concentrations of homocysteine with formation of the thiazanecarboxylic acid. The relative rates of methionine synthesis from serine and tetrahydropteroylglutamic acid–

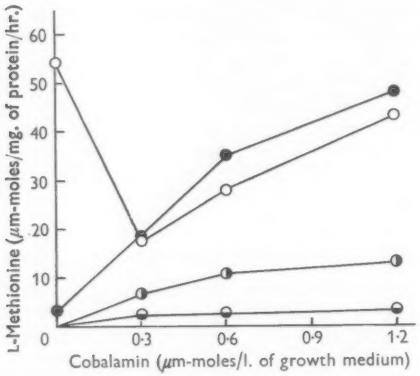


Fig. 1. Effect of varying concentrations of cobalamin in the growth medium on methionine synthesis by extracts of acetone-dried organisms. Standard reaction mixture was used, except that L-serine was omitted and hexose diphosphate was 10 μmoles. Source of enzyme: dialysed extracts of organisms grown in the presence of the stated concentrations of cobalamin and equivalent to 7 mg. of protein for the experiments with serine alone and to 9 mg. in other cases. Additions to reaction mixture: L-serine (10 μmoles), tetrahydropteroylglutamic acid and tetrahydropteroylglutamic acid–CH_2O (each 3·6 μmoles). Incubated for 4 hr. ○, L-Serine; ●, L-serine plus tetrahydropteroylglutamic acid; ◑, tetrahydropteroylglutamic acid–CH_2O; ◒, tetrahydropteroylglutamic acid.

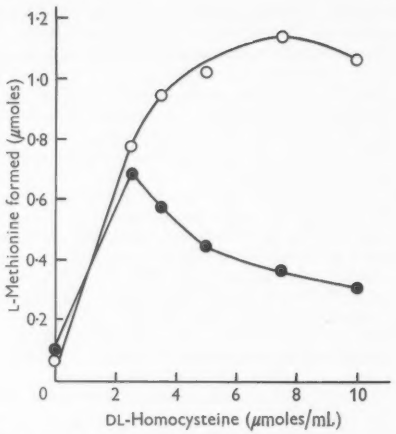


Fig. 2. Effect of concentration of DL-homocysteine on methionine synthesis. Standard reaction mixture was used, with the omission of L-serine, and, in the experiment with tetrahydropteroylglutamic acid–CH_2O, pyridoxal phosphate; DL-homocysteine was present as indicated. Source of enzyme: dialysed extract (equivalent to 10 mg. of protein) of organisms grown with cobalamin. Incubated for 3 hr. Additions to reaction mixture: ○, L-serine, 10 μmoles; ●, tetrahydropteroylglutamic acid–CH_2O, 3·3 μmoles.

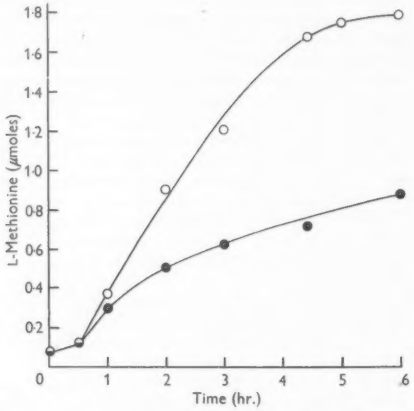


Fig. 3. Rate of methionine synthesis with different C_1-donor systems. Standard reaction mixture (L-serine omitted), but with DL-homocysteine 15 and 5 μmoles for the experiments with L-serine and tetrahydropteroylglutamic acid–formaldehyde respectively, was used. Source of enzyme: dialysed extract (equivalent to 10 mg. of protein) of cobalamin-grown organisms. C_1-donor systems: ○, serine (10 μmoles) plus tetrahydropteroylglutamic acid (1 μmole); ●, tetrahydropteroylglutamic acid–formaldehyde (3·3 μmoles).

formaldehyde respectively with appropriate concentrations of homocysteine gave a truer comparison (Fig. 3); the rate with tetrahydropteroylglutamic acid–formaldehyde is about one-half of that with serine.

Recovery of added methionine. Methionine (0·5 μmole) was quantitatively recovered if added to the standard reaction mixture (serine omitted) together with tetrahydropteroylglutamic acid–formaldehyde; this was true whether or not the original growth medium contained cobalamin. Breakdown of methionine (whether by reversal of the reaction under investigation or by some other mechanism) cannot therefore explain the results obtained.

Catalytic action of tetrahydropteroylglutamate

Residual folic acid coenzyme in dialysed enzymic extracts was removed by treatment with Dowex-1 resin. An extract of cobalamin-grown organisms so treated can be reactivated (serine as C_1 donor) by tetrahydropteroylglutamic acid (Table 1). That tetrahydropteroylglutamic acid is acting catalytically is shown by the following quantitative considerations. At the lowest concentration of tetrahydropteroylglutamic acid added (0·3 μmole) 1·2 μmoles of methionine was formed; the effective concentration of tetrahydropteroylglutamic acid would, however, be only 0·1 μmole since it was only 70 % pure and it may be assumed that only one-half of this is the correct stereoisomer.

With serine as C_1 donor relatively high concentrations of tetrahydropteroylglutamic acid reduced methionine synthesis (20 % by 6 μmoles and 70 % by 12 μmoles, compared with the synthesis obtained with 1 μmole). However, with tetrahydropteroylglutamic acid–formaldehyde as direct substrate methionine synthesis (compared with that given by 3·7 μmoles) decreased only 30 % on addition of 15 μmoles.

Table 1. *Activation of enzyme from cobalamin-grown organisms by tetrahydropteroylglutamic acid*

Standard reaction mixture (except that DL-homocysteine was 15 μmoles), containing dialysed, Dowex-1-treated extract of cobalamin-grown organisms equivalent to 9 mg. of protein, was used. L-Serine was C_1 donor. Incubated for 5 hr.

Tetrahydropteroylglutamic acid added (μmole)	L-Methionine formed (μmoles)
0	0·06
0·3	1·36
0·4	1·84
0·6	2·30
1·0	2·80

Other requirements for methionine synthesis

The effect of omitting the various components of the standard reaction mixture was tested with dialysed, Dowex-1-treated enzymic extracts of cobalamin-grown organisms with both serine and tetrahydropteroylglutamic acid–formaldehyde as C_1 donor (Table 2). Apart from the substrates and energy source (hexose diphosphate) there was strong evidence in both cases of requirements for ATP, DPN and Mg^{2+} ions; DPN could not be replaced by TPN, which was inhibitory when added as well as DPN. Similar requirements were found by Cross & Woods (1954) with their extract of

Table 2. *Requirements for methionine synthesis by partly purified extracts of cobalamin-grown organisms*

Standard reaction mixture was used, omitting L-serine, and with DL-homocysteine present [15 μmoles (column *a*) and 10 μmoles (column *b*)]; other modifications were as indicated. When tetrahydropteroylglutamic acid and formaldehyde were both present (column *b*) they were added (3·3 μmoles) as a preformed equimolar mixture. For column *a* the amount of tetrahydropteroylglutamic acid was 1 μmole. L-Serine (when present), 10 μmoles. Incubated for 2·5 hr.

C_1 donor	L-Methionine formed (μmole): L-Serine	L-Methionine formed (μmole): Tetrahydropteroylglutamic acid–formaldehyde
Modifications to reaction mixtures	(*a*)	(*b*)
None	0·87	0·37
No L-serine	0·09	—
No formaldehyde	—	0·18
No tetrahydropteroylglutamic acid	0·08	0·06
No DL-homocysteine	0·08	0·11
No DPN	0·17	0·13
TPN (1 μmole) instead of DPN	0·09	0·14
DPN plus TPN	0·23	0·24
No ATP	0·20	0·11
No hexose diphosphate	0·15	0·11
No pyridoxal phosphate	0·31	0·40
No Mg^{2+} ions	0·22	0·22
None	0·57	0·40
Plus cobalamin (50 μmg.)	0·58	0·42

alumina-ground organisms and by Szulmajster & Woods (1955, 1960) with extracts of acetone-dried organisms of the present strain grown without cobalamin.

Omission of pyridoxal phosphate resulted in a sharp though variable drop in methionine synthesis (60–100% with different preparations) when serine was the C_1 donor (Table 2). In previous work (Cross & Woods, 1954; Szulmajster & Woods, 1960) the effect of this factor, though clear, was also somewhat variable. This may be due to the rapid rate of transformation of serine into glycine (0·33 μmole/min.; Fig. 4) compared with the rate of methionine formation (0·006 μmole/min.; Fig. 3). During the relatively long incubation required for methionine synthesis any trace of pyridoxal phosphate remaining in the extracts, or released during the incubation, might mediate the formation of some C_1 units from serine.

By contrast, when tetrahydropteroylglutamic acid–formaldehyde was C_1 donor, no requirement for pyridoxal phosphate was found (Table 2); the possibility that it is concerned in a less easily dissociated form in the activation of homocysteine cannot be excluded. It is clear, however, that the carbon atom of formaldehyde need not be converted into the β-C of serine before its conversion into the methyl carbon atom of methionine. Furthermore, the addition of glycine (1 μmole) did not increase methionine synthesis with tetrahydropteroylglutamic acid–formaldehyde as C_1 donor as might be expected if the formation of serine was an obligatory step.

Effect of cobalamin. The enzymic extract used in the experiments of Table 2 was from organisms grown with cobalamin and this vitamin had, as might be expected, no effect on methionine synthesis. However, this was also the case with preparations from organisms grown without cobalamin (Table 4); the free vitamin promoted no synthesis of methionine with serine as C_1 donor and tetrahydropteroylglutamic acid as cofactor.

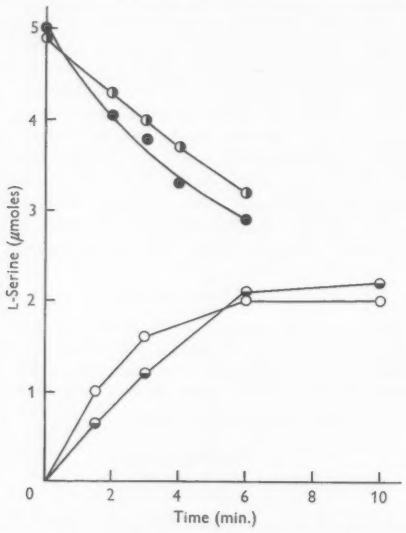


Fig. 4. Synthesis and breakdown of serine by enzymic extracts. Synthesis: Standard reaction mixture, with L-serine and DL-homocysteine omitted and glycine (10 μmoles) and tetrahydropteroylglutamic acid–formaldehyde (5 μmoles) added, was used. Dialysed enzymic extracts each contained 8·5 mg. of protein and were derived from organisms grown with (◒) and without (○) cobalamin. Breakdown: Reaction mixture contained (in 2 ml.): L-serine (5 μmoles), tetrahydropteroylglutamic acid (5 μmoles), pyridoxal phosphate (1 μmole) and potassium phosphate, pH 7·8 (200 μmoles). Dialysed enzymic extracts each contained 5 mg. of protein and were derived from organisms grown with (◑) and without (●) cobalamin.

Degradation and synthesis of serine

Since it is probable that the reaction generating the C_1 unit is the conversion of serine into glycine it was necessary to determine whether growth on cobalamin affected this stage of the over-all synthesis of methionine. The present enzymic extracts catalysed both the breakdown of serine and its synthesis from glycine and tetrahydropteroylglutamic acid–formaldehyde. The assay of serine is technically more satisfactory than that of glycine, which was not attempted.

Table 3. *Synthesis of serine by partly purified extracts*

The reaction mixture contained (in 2 ml.): glycine, 10 μmoles; tetrahydropteroylglutamic acid–formaldehyde, 5 μmoles; potassium phosphate, pH 7·8, 200 μmoles; pyridoxal phosphate, 1 μmole; dialysed, Dowex-1-treated extract of organisms grown either with or without cobalamin equivalent to 6 mg. of protein. Incubated for 6 min.

Modifications to reaction mixture	Serine formed (μmoles)* with extract of organisms with cobalamin (in growth medium)	
	Present	Absent
None	2·6	2·7
No glycine	0	0
No tetrahydropteroylglutamic acid–formaldehyde	0	0
No pyridoxal phosphate	0·1	0·15
Plus $MnSO_4$ (1 μmole)	1·3	1·0
Albumin† (6 mg.) instead of enzymic extract	0	—

* Values are corrected for a blank of 0·2 μmole.

† Bovine serum albumin fraction V (The Armour Laboratories, Hampdon Park, Eastbourne).

The synthesis of serine by dialysed enzymic extracts (Dowex-1-treated) was rapid (Fig. 4) and dependent (Table 3) upon the presence of tetrahydropteroylglutamic acid (as tetrahydropteroylglutamic acid–formaldehyde), which was used equally well whether or not the organisms had been cultivated in the presence of cobalamin (Fig. 4). The degradation of serine also required tetrahydropteroylglutamic acid and again occurred irrespective of the presence of cobalamin in the original growth medium (Fig. 4). It was concluded that the function of tetrahydropteroylglutamic acid which is related to cobalamin concerns a later phase in the over-all synthesis of methionine.

The synthesis of serine also requires pyridoxal phosphate and is partly inhibited by Mn^{2+} ions (Table 3); DL-homocysteine (5 μmoles/ml.) reduced synthesis by 70%, possibly due to the removal of C_1 units by the formation of 1:3-thiazane-4-carboxylic acid. Although the synthesis of serine from glycine and tetrahydropteroylglutamic acid–formaldehyde is very rapid it is certainly an enzymic reaction; there was no synthesis when albumin was substituted for the extract of organisms (Table 3). There was no requirement for ATP, DPN, hexose diphosphate or Mg^{2+} ions.

Factor present in cobalamin-grown organisms

The results so far described point to the presence in cobalamin-grown organisms of a factor which permits the utilization of tetrahydropteroylglutamic acid as coenzyme in the methylation of homocysteine. Further experiments were devoted to the simple properties of this factor.

Table 4. *Assay of the factor present in cobalamin-grown organisms*

Standard reaction mixture supplemented with tetrahydropteroylglutamic acid (2 μmoles) and containing dialysed extract (equivalent to 5 mg. of protein) of organisms grown without cobalamin (extract WC). Extract C (equivalent to 1·8 mg. of protein) was prepared in the usual way from organisms grown with cobalamin, and the protamine ppt. and supernatant (each 0·2 ml.) were derived from it (see text). Incubated for 3 hr.

Source of factor	Modifications to reaction mixture	L-Methionine formed (μmoles)
None	No tetrahydropteroylglutamic acid	0·55
None	None	0·08
Cobalamin (50 μmg.)	None	0·10
Extract C	None	1·36
Extract C	No extract WC	0·11
Protamine ppt.	None	1·14
Protamine supernatant	None	0·13
Protamine ppt. plus protamine supernatant	None	1·16

Detection and assay. Amounts of enzymic extract of cobalamin-grown organisms insufficient alone to catalyse appreciable methionine synthesis enabled extracts of organisms grown without cobalamin to form methionine with serine as C_1 donor and tetrahydropteroylglutamic acid as cofactor (Table 4). Quantitative assay could be made in two ways: (*a*) by using a dialysed extract (organisms grown without cobalamin) treated with Dowex-1 resin to remove residual folic acid coenzyme or (*b*) by using extracts untreated with Dowex-1 and relying upon the fact that any effect of natural folic acid coenzyme is inhibited by the tetrahydropteroylglutamic acid which is added. The second method was simpler and proved reliable in practice; it was used as a routine in the work which follows. Samples of the material from cobalamin-grown organisms in amounts insufficient for methionine synthesis were added to the standard reaction mixture containing tetrahydropteroylglutamic acid and the normal quantity of enzymic extract from organisms grown without cobalamin used to demonstrate methionine synthesis (Table 4). Controls in which one or other of the two forms of extract was omitted were included in each experiment.

Properties of the factor. The experimental material was the initial aqueous extract of acetone-dried organisms harvested after growth on cobalamin. The factor was non-diffusible and was destroyed by heating to 65° for 3 min. It was stable to weak alkali (pH 10·4, 30 min. at 0°) but was labile in dilute acid; in 30 min. at 0° 30% activity was lost at pH 4·2, and 45% at pH 3·6. Freezing and thawing did not change the activity. When treated with Dowex-1 resin (as for the removal of residual folic acid coenzyme) about 40% of the activity was lost.

Partial purification. The crude extract (8 ml.) was treated with 2% (w/v) protamine sulphate (1·2 ml.). The supernatant after centrifuging contained no factor, but the precipitate (suspended in 8 ml. of 0·05M-potassium phosphate, pH 7·8) had 85% of the original activity (Table 4). This was not increased by adding the supernatant as well. Controls in which protamine sulphate (0·5 mg./ml.) was added to the reaction mixture showed that this substance did not affect methionine synthesis.

The active material was extracted from the protamine precipitate by rubbing it up with 0·1M-potassium phosphate, pH 7·8 (5 ml.). After centrifuging to remove insoluble material the supernatant was dialysed for 12 hr. against more dilute (0·05M) potassium phosphate, pH 7·8; a fine white precipitate formed rapidly and this contained about 50% of the total original activity (fraction I). The response of enzymic extracts of organisms grown without cobalamin to graded amounts of this

material (suspended in buffer) was not quite linear over the range tested (Table 5); fraction I itself, in the quantities used, did not promote methionine synthesis.

Fraction I was brought into solution by treating the suspensions with an equal volume of 0·1 M-sodium pyrophosphate, pH 9·1, for 15 min. at 37°. The pyrophosphate was then removed by addition of two molar equivalents of magnesium sulphate and centrifuging. The resulting solution (fraction II) had about 75% of the activity of fraction I (Table 6) and this was not appreciably diminished by dialysis against distilled water for 14 hr. The amount of fraction II added in the experiments of Table 6 contained 0·2 mg. of protein (280 mμ/260 mμ ratio 1·3), some of which was probably protamine.

Cobalamin content. The soluble fraction II was assayed for cobalamin with *Euglena gracilis* and was found to contain 21 μμg./mg. of protein. The value was not changed by dialysis (free cobalamin was diffusible under the conditions used) and it was concluded that the cobalamin present was in a bound state. The amount of the vitamin added with fraction II in the experiments of Table 6 was thus about 1·6 μμM, which is of the same order of magnitude as that (7 μμM) required for methionine synthesis by ultrasonic extracts of an auxotrophic strain of *E. coli* requiring cobalamin for growth (Helleiner, Kisliuk & Woods, 1957).

At the present stage of purification little significance can be attached to the presence of bound cobalamin in the active fraction; it may or may not be associated with a contaminating protein since the organisms had been grown originally with added vitamin.

Glycine as one-carbon-atom donor

Glycine replaced serine as C_1 donor but was only about one-third as active as the latter (Table 7). With a dialysed extract (Dowex-1-treated) of organisms grown without cobalamin the enzyme was activated either by a heated extract of the acetone-dried organisms or by tetrahydropteroylglutamic acid plus partly purified (fraction I) extract of organisms grown with cobalamin (Table 7). The position was therefore the same as with serine as donor.

DISCUSSION

Role of cobalamin

Growth on a medium containing cobalamin is essential if extracts of acetone-dried *Escherichia coli* are to synthesize methionine either with tetrahydropteroylglutamic acid as carrier of single-carbon-atom units from serine or with the formal-

Table 5. *Effect of partly purified extract of cobalamin-grown organisms on methionine synthesis by extracts of organisms grown without cobalamin*

Standard reaction mixture, with tetrahydropteroylglutamic acid (1 μmole) added, was used. L-Serine was C_1 donor. Fraction I (see text) was derived from extract of cobalamin-grown organisms. Extract WC, dialysed extract of organisms grown without cobalamin (equivalent to 5·2 mg. of protein), was added as shown. Incubated for 3 hr. +, Present; −, absent.

Additions		L-Methionine formed (μmoles)
Extract WC	Fraction I (ml.)	
+	0	0·12
+	0·05	0·51
+	0·1	0·77
+	0·2	1·20
−	0·1	0·10

Table 6. *Assay of fractions derived from extracts of cobalamin-grown organisms*

Standard reaction mixture supplemented with tetrahydropteroylglutamic acid (1 μmole), and containing dialysed extract (equivalent to 6 mg. of protein) of organisms grown without cobalamin, was used. Fraction I (0·1 ml.) and an equivalent volume of fraction II were derived from it as described in the text; dialysis was at 3° for 12 hr. against distilled water. Incubated for 3 hr.

Source of fraction	L-Methionine formed (μmole)
None	0·10
Fraction I	0·88
Fraction II	0·64
Fraction II, dialysed	0·60

Table 7. *Comparison of glycine and serine as precursors of the methyl group of methionine*

Standard reaction mixture (without L-serine), containing dialysed, Dowex-1-treated extract of organisms grown without cobalamin (equivalent to 5 mg. of protein) was used. L-Serine or glycine (each 10 μmoles) was C_1 donor; tetrahydropteroylglutamic acid (when present), 1 μmole. Fraction I (0·1 ml.) was derived (see text) from extract of cobalamin-grown organisms. For details of the heated extract of *E. coli* (0·1 ml.) see Materials and Methods section. Incubated for 3 hr.

Cofactors added	L-Methionine formed (μmole)	
	With L-serine	With glycine
None	0·07	0·05
Tetrahydropteroylglutamic acid	0·05	0·05
Tetrahydropteroylglutamic acid plus fraction I	0·47	0·17
Heated extract of *E. coli*	0·36*	0·15*

* These values were reduced to 0·05 if serine and glycine respectively were omitted.

dehyde derivative of this compound (tetrahydropteroylglutamic acid–formaldehyde) as direct donor of C_1 units. Extracts from organisms grown without cobalamin are not activated by the free vitamin. It would appear likely that the treatment with acetone destroys an enzyme system(s) which converts cobalamin into a metabolically active form; ultrasonic extracts of both the present strain of *E. coli* and an auxotroph requiring cobalamin for growth respond to free cobalamin and in its presence they can use tetrahydropteroylglutamic acid as C_1 carrier or tetrahydropteroylglutamic acid–formaldehyde as direct C_1 donor (Helleiner & Woods, 1956; Helleiner *et al.* 1957; Woods, 1958).

Whilst this paper was in preparation there has been a brief report (Hatch, Takeyama & Buchanan, 1959) of the probable presence of a cobalamin-containing enzyme in a system synthesizing methionine isolated from a cobalamin auxotroph of *E. coli*.

The interconversion of serine and glycine by extracts of acetone-dried organisms is dependent on tetrahydropteroylglutamic acid, which is used whether or not the organisms have been grown with cobalamin. The effect of cobalamin must therefore be sought at some other stage of methionine synthesis than the initial activation of the C_1 donor. The nature of the effect is not made clear by the present experiments but a number of possibilities may be considered.

(1) *A derivative of cobalamin might convert tetrahydropteroylglutamic acid into a coenzyme form.* This seems unlikely because methionine synthesis by crude enzymic extracts (presumably containing a coenzyme form of folic acid) is completely inhibited by tetrahydropteroylglutamic acid; furthermore, a heated extract of *E. coli*, which also probably contains such a coenzyme form (Szulmajster & Woods, 1955, 1960), did not overcome the inhibition. Any coenzyme formed from tetrahydropteroylglutamic acid by the mediation of cobalamin would either have to be different from that found in normal cell extracts or it would have to be assumed that virtually all the added tetrahydropteroylglutamic acid was converted into a coenzyme form. However, heated extracts of cobalamin-grown organisms also did not overcome the inhibition by tetrahydropteroylglutamic acid. The alternative proposition is also unlikely since high concentrations of tetrahydropteroylglutamic acid do not markedly reduce methionine synthesis by extracts of cobalamin-grown organisms (i.e. when tetrahydropteroylglutamic acid is the carrier).

(2) *Cobalamin may be required for the synthesis of proteins concerned in methionine synthesis.* Certain of the properties of the material isolated from cobalamin-grown organisms and required for the utilization of tetrahydropteroylglutamic acid in methionine synthesis resemble those of a protein (thermolability, non-diffusibility). It is possible therefore that cobalamin promotes the synthesis of a protein specifically required for the utilization of tetrahydropteroylglutamic acid. This would assume that the active factor is not itself a cobalamin derivative; the evidence on this point is so far inconclusive.

(3) *Cobalamin may act as a non-specific stimulator of a number of enzyme reactions.* Dubnoff & Bartron (1956) have given evidence for this as a general function of cobalamin. The following points illustrate important differences between the effect on methionine synthesis and such non-specific stimulations.

First, the metabolism of serine is unaffected by growth of the organism on cobalamin, whereas it is essential for methionine synthesis with tetrahydropteroylglutamic acid as cofactor.

Secondly, it is clear that accessory enzymes (such, for example, as are necessary for the formation of reduced DPN) are not limiting in extracts of organisms grown without cobalamin. For instance, crude enzymic extracts (containing natural folic acid coenzyme) synthesize methionine in the absence of tetrahydropteroylglutamic acid just as well as do extracts of cobalamin-grown organisms in its presence.

(4) *Possible breakdown of methionine.* It was possible that under the conditions imposed by the presence of tetrahydropteroylglutamic acid there was decomposition of methionine as rapidly as it was formed, and that growth on cobalamin in some way prevented this. However, it was found that added L-methionine could be completely recovered from systems containing enzymic extracts of both types.

(5) *A cobalamin derivative may act catalytically in the reduction of an intermediate at the hydroxymethyl level of oxidation to a methyl group.* This hypothesis presupposes that the active factor is a cobalamin derivative and this is not proved; such a role for cobalamin is favoured by its structural similarity to metalloporphyrins. It is suggested that homocysteine condenses with the ring-closed form of hydroxymethyltetrahydropteroylglutamic acid ($N^{5:10}$-methylenetetrahydropteroylglutamic acid) to form the derivative (I) (Fig. 5). The cobalamin derivative might then transfer electrons from reduced DPN to (I), resulting in a reductive cleavage of the latter to methionine and tetrahydropteroylglutamic acid (Fig. 5). Such a reduction system might be considered analogous to the reverse of the electron transfer from flavoproteins to cytochrome; that is, a cobalamin derivative instead of cytochrome and a folic acid protein instead of flavoprotein.

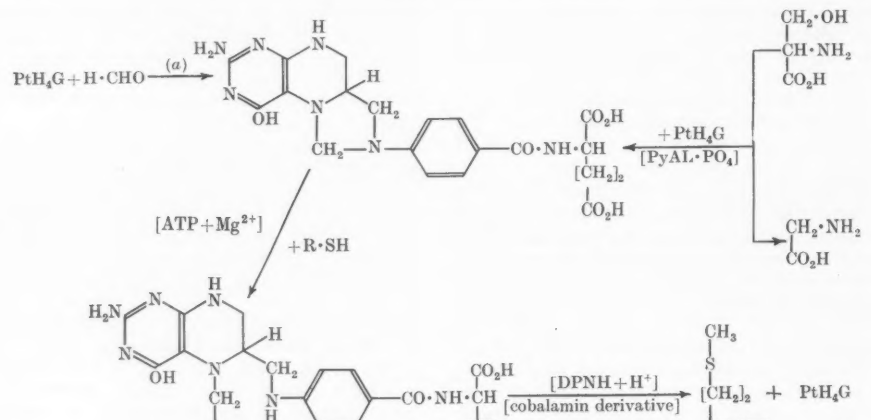
Extracts of organisms (grown without cobalamin) carry out methionine synthesis by a pathway which is inhibited by tetrahydropteroylglutamic acid; the latter may therefore be acting as an inhibitory analogue of the naturally occurring folic acid coenzyme. Growth on a medium containing cobalamin may endow the extracts with an alternative mechanism for the reduction of the hydroxymethyl group in which tetrahydropteroylglutamic acid can act as the carrier of this group. Davis & Mingioli (1950) described mutants of *E. coli* that require either methionine or cobalamin for growth. It is possible that cobalamin acts by making possible an alternative pathway for the reduction of the hydroxymethyl group and not by providing the product of the reaction blocked by the mutation; in this case the metabolic lesion in these mutants would be at a step in the normal synthesis of methionine rather than (as is generally supposed) in the synthesis of cobalamin itself. The formation of the methyl group of methionine in prototrophic strains of *E. coli* may be independent of cobalamin, as it appears to be in higher plants for which cobalamin is not an essential metabolite.

(6) *A cobalamin derivative may be required to activate amino acids.* Wagle, Mehta & Johnson (1958*a*, *b*) have given evidence for this role of cobalamin in animal metabolism, but have later (Mehta, Wagle & Johnson, 1959) withdrawn the suggestion. Although it is possible to suppose that in methionine synthesis cobalamin might be required for the formation of an adenosine monophosphate–homocysteine derivative which is the actual acceptor of the methyl group, it is difficult at present to reconcile such an hypothesis with the fact that cobalamin is required only when tetrahydropteroylglutamic acid is the carrier of C_1 units and not when natural folic acid coenzyme is available.

A second possibility is that the cobalamin derivative might be required for the activation of glutamate and thus for the formation of polyglutamyl derivatives of tetrahydropteroylglutamic acid, which may be more effective than the free compound as C_1-unit carriers. Polyglutamyl derivatives of pteroylglutamic acid are more effective than tetrahydropteroylglutamic acid in catalysing the breakdown of serine by extracts of *Clostridium sticklandii* (Wright & Stadtman, 1956), and an *N*-formylpteroyltriglutamic acid partially replaces natural folic acid coenzyme in the present system (Szulmajster & Woods, 1960). The discussion of possibility (1) above also applies to this suggestion and militates against it.

Other considerations

Recent investigations of methionine synthesis in liver preparations (Nakao & Greenberg, 1958; Stevens & Sakami, 1958) show tetrahydropteroylglutamic acid to be an effective cofactor; no effect



(I)

Fig. 5. Hypothetical scheme of reaction. R·SH is homocysteine [HS·CH_2·CH_2·$CH(NH_2)$·CO_2H]. PtH_4G and PyAL·PO_4 represent tetrahydropteroylglutamic acid and pyridoxal phosphate respectively; (*a*) is a non-enzymic reaction. DPNH, Reduced DPN.

of cobalamin was reported. The other requirements were similar to those in the present work except that TPN was more active than DPN.

The mode of action of ATP is not known; it may react with homocysteine forming either *S*-adenosylhomocysteine or homocysteine–adenosine monophosphate, either of which might be the final acceptor of a reduced C_1 unit rather than free homocysteine. It probably also serves as a source of ADP for glycolysis. A requirement for inorganic phosphate for methionine synthesis was shown by Szulmajster & Woods (1960); phosphate was always added to the reaction mixture in the present work and the point was not further investigated.

The relative inactivity of glycine compared with serine as an ultimate source of the methyl group was also observed with suspensions of the organism (Gibson & Woods, 1960). However, conditions for optimum utilization of glycine by the enzymic extracts were not studied in detail. It is also possible that some of the C_1 units formed from glycine condense with unchanged glycine to form serine; glycine would thus be competing with the system synthesizing methionine for the available C_1 units.

SUMMARY

1. Aqueous extracts of acetone-dried *Escherichia coli* PA15 (a serine or glycine auxotroph) synthesize methionine from homocysteine and serine. Tetrahydropteroylglutamic acid appears to inhibit the function of the natural folic acid coenzyme present in the extracts.

2. Tetrahydropteroylglutamic acid will, however, itself serve as cofactor, but only provided that the organisms have been grown on a medium containing cobalamin. Addition of cobalamin to the enzymic extract has no effect.

3. A non-diffusible, heat-labile material which promotes methionine synthesis in the presence of tetrahydropteroylglutamic acid has been obtained from extracts of organisms grown with cobalamin and partly purified. The purified fraction still contains a significant amount of cobalamin.

4. The reversible transformation of serine into glycine is catalysed by tetrahydropteroylglutamic acid whether or not the organisms have been grown with cobalamin.

5. With L-serine as the C_1 donor the other requirements for methionine synthesis are homocysteine, hexose diphosphate, adenosine triphosphate, diphosphopyridine nucleotide, pyridoxal phosphate and Mg^{2+} ions. With the formaldehyde derivative of tetrahydropteroylglutamic acid as donor pyridoxal phosphate is not required.

6. Serine is not on the pathway of synthesis of the methyl group of methionine from formaldehyde.

7. Glycine has one-third of the activity of serine as C_1 donor when tested under the same conditions.

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Studies on Sulphatases

28. PREPARATION OF SUBSTRATES FOR THE ASSAY OF GLYCOSULPHATASE*

By A. G. LLOYD†
Department of Biochemistry, University of Wales, Newport Road, Cardiff

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Glucose monosulphate, used as assay substrate for the study of the enzyme glycosulphatase, has been prepared by the direct action of chlorosulphonic acid or pyridine–sulphur trioxide reagent on glucose in pyridine solution (see Dodgson & Spencer, 1957). With material prepared in this way, Dodgson & Spencer (1954), during a study of the glycosulphatase of the marine mollusc *Littorina littorea*, observed anomalies in the behaviour of the enzyme toward increasing substrate concentration, which were attributed to the presence of one or more sulphated contaminants in the substrate preparation. Attempts to remove these contaminating materials were unsuccessful, and further kinetic studies were abandoned. Lloyd (1959*a*) showed that monosaccharide sulphate esters prepared by the direct action of chlorosulphonic acid invariably consist of mixtures of mono- and di-sulphate esters, together with small amounts of unchanged monosaccharides. For this reason methods were evolved for the definitive synthesis of potassium glucose 6-*O*-sulphate and potassium *N*-acetylglucosamine 6-*O*-sulphate with chlorosulphonic acid as sulphating agent. As chlorosulphonic acid yields reaction mixtures contaminated with chloride, which can be removed only with difficulty, the method is not ideally suited to the preparation of enzyme substrates for routine studies.

In the present report methods are described for the synthesis of monosaccharide monosulphate esters by a modification of the method due to Duff (1949). A preliminary account of part of this work has already been given (Lloyd, 1959*b*).

MATERIALS AND METHODS

Monosaccharides and derivatives. D-Glucose, D-galactose and D-glucosamine were commercial preparations (British Drug Houses Ltd.). D-Galactosamine was prepared from potassium chondroitin sulphate (Dodgson, Lloyd & Spencer, 1957) by the method of Roseman & Ludowieg (1954). The procedure described by the latter workers was used for the *N*-acetylation of both the glucosamine and galactosamine preparations. Several authentic carbohydrate sulphates were prepared by the chlorosulphonic acid procedure (Lloyd, 1959*a*). Potassium glucose 6-*O*-sulphate was obtained after sulphating 1:2:3:4-tetra-*O*-acetyl-D-glucose (Reynolds & Evans, 1942), potassium galactose 6-*O*-sulphate after sulphating 1,2:3,4-di-(*O*-*iso*propylidene)-D-galactose and potassium *N*-acetylglucosamine 6-*O*-sulphate after sulphating 1:3:4-tri-*O*-acetyl-*N*-acetyl-D-glucosamine (Anderson & Percival, 1956).

Sulphation with pyridine–sulphur trioxide reagent. The monosaccharide (0·05 mole) was dissolved in 100 ml. of dry pyridine in the presence of 10 g. of Drierite (L. Light and Co. Ltd.) at 70°. The mixture was cooled to 37° and stirred mechanically during the addition of 0·10 mole of pyridine–sulphur trioxide reagent (Baumgarten, 1926). The reaction mixture was stirred at 37° for 12 hr., cooled to 20° and kept for 3hr., when the crude ester sulphate separated as an oily lower layer. The upper pyridine layer was removed by decantation and the oil dissolved in the minimum amount of water at room temperature with vigorous stirring. Drierite was removed by filtration and the clear filtrate stirred at room temperature for 2 hr. The solution was cooled to 2°, and a suspension of 40 g. of $Ba(OH)_2$ in 100 ml. of water, previously cooled to 2°, was added with constant stirring over a period of 10 min. Precipitated $BaSO_4$ was removed by centrifuging at 3000 *g* and 0° for 15 min. Ba^{2+} ions were precipitated from the alkaline supernatant solution by the addition of solid CO_2 over a period of 30 min. Precipitated $BaCO_3$ was removed by centrifuging as before. The clear supernatant solution was distilled under reduced pressure in a rotary evaporator at 35° to remove pyridine, water being added continuously to avoid concentration. The solution was finally concentrated to 20 ml. by distillation *in vacuo* at 30°.

Removal of the greater part of the unchanged monosaccharide was achieved as follows. The concentrated solution containing the crude monosaccharide sulphate was passed through a Dowex 50 column (H^+ form; 100–200 mesh; cross-linkage ×4; 30 cm. ×2 cm.) at 2° at a flow rate of 1 ml./min. The acid eluate and washings were combined (40 ml.) and treated with a saturated ethanolic solution of brucine (20 ml.) at room temperature. The mixture was then concentrated to dryness under reduced pressure at 35°, a precipitate of brucine which appeared during the concentration procedure being removed by filtration. The residue was dissolved in the minimum amount of water at 50° with vigorous stirring, cooled to 25° and absolute ethanol (about 250 ml.) added. The mixture was cooled to 5° and kept for 2 hr. The resulting precipitate was filtered and recrystallized twice from aqueous ethanol as described above.

For conversion into the potassium salt the brucine derivative was dissolved in the minimum amount of water and passed through a Dowex 50 column (H^+ form as before).

* Part 27: Dodgson & Powell (1959).

† Empire Rheumatism Council Research Fellow.

The acid eluate and washings were adjusted to pH 7·4 with 0·05M-KOH and then concentrated to small volume by distillation under reduced pressure at 35°. The potassium salt was precipitated by the addition of 5 vol. of ethanol, the precipitate was collected by filtration and, after being washed with absolute ethanol and ether, finally dried *in vacuo* at 30° over $CaCl_2$.

Paper chromatography. The homogeneity of the reaction products was checked by descending chromatography on Whatman no. 3MM paper for 48 hr. at 20° with butan-1-ol–acetic acid–water, 50:12:25 (by vol.). Spots were detected by spraying with silver nitrate (Trevelyan, Procter & Harrison, 1950) or aniline hydrogen phthalate (Partridge, 1949). Hexosamines and derivatives were demonstrated by spraying with Elson–Morgan reagents (Partridge, 1948). For the detection of sulphuric acid esters the chromatograms were sprayed with 10% (v/v) of perchloric acid in ethanol, followed by heating for 5 min. at 80–85° in a moist (water vapour) atmosphere. The inorganic sulphate so released was then detected by spraying with $BaCl_2$ followed by sodium rhodizonate (Burma, 1953).

Filter-paper electrophoresis. Zone electrophoresis on filter-paper strips (32 cm. in length) was carried out in horizontal tanks with a multipoint paper support. Several volatile buffer systems, chosen to avoid time-consuming desalting procedures, were examined during initial stages of the study. Best separations of monosaccharide monosulphate from the contaminating disulphate and parent compounds were obtained after electrophoresis in ammonium acetate–acetic acid buffer (0·1M; pH 4·0). For analytical purposes, Whatman no. 3MM filter paper was employed, the applied potentials being varied in the range 110–400 v over periods of 2–12 hr.

Filter-paper electrophoresis was also employed for small-scale preparative procedures on Whatman no. 17 or Whatman Seed Test papers. Before use, the sheets were washed by downward irrigation with 0·01N-HCl for 48 hr., with water for 48 hr. and finally with the buffer to be used for the separation for a further 24 hr. The sheets were finally dried in a warm air stream.

The material to be separated was applied as a narrow zone across the width of the thick filter-paper strip (32 cm. × 11 cm.) from a self-filling micropipette. On Whatman no. 17 paper it was possible to apply a maximum of 100 mg./strip, and on Seed Test paper this could be increased to 175 mg./strip. However, with the latter there was considerable variation in the efficiency of the separation since marked zone-spreading occurred with some samples of the paper. Contact between the ends of the thick filter-paper strip and the electrolyte chambers of the electrophoresis apparatus was made with Whatman no. 1 wicks.

After passage of the current the damp filter paper was removed from the tank and placed on a clean glass plate. A piece (32 cm. × 11 cm.) of Whatman no. 1 paper was placed on top of the strip and the two were pressed evenly together by applying gentle pressure with a soft rubber roller. As the upper strip absorbed moisture from the lower one, traces of material from the separated zones were transferred to it. After the upper strip had been sprayed, the thick filter-paper strip could be returned to the tank if separation was found to be incomplete.

The appropriate regions of the electrophoresis strip were then cut out, clamped between glass plates and eluted with water. The eluates were concentrated to dryness by freeze-drying over KOH. The residue was taken up in the minimum amount of water, passed through a Dowex 50 column (H^+ form; 100–200 mesh), and the acid eluate and washings were pooled and adjusted to pH 7·4 with 0·05M-KOH and concentrated *in vacuo*. Ethanol (5 vol.) was added to the solution and the precipitated material allowed to flocculate. The precipitate was collected by centrifuging at 2000 *g* and 0° for 10 min., washed with absolute ethanol and ether and finally dried *in vacuo* at 30° over $CaCl_2$.

Cellulose-column electrophoresis. Zone electrophoresis was carried out in externally cooled columns at 4° (Porath, 1956). On an analytical scale the column dimensions were 100 cm. × 1·5 cm., and for preparative purposes the dimensions were 80 cm. × 3 cm. Packing of the ethanolysed cellulose powder was performed as described by Flodin & Kupke (1956), after removal of gases from the buffer slurry at 10° under reduced pressure. The cellulose bed was then washed with 5 hold-up vol. of buffer before use.

When electrophoresis was complete the column was eluted with buffer at 40 ml./hr. (analytical scale) or at 100 ml./hr. (preparative scale). The position of displaced zones was determined quantitatively by the anthrone procedure (Fairbairn, 1953). Fractions corresponding to homogeneous elution peaks were pooled, concentrated to small volume by distillation *in vacuo* at 35° and, after freeze-drying over KOH, treated as described for the isolation of material after zone electrophoresis on thick paper.

Analysis. All samples were dried at 40° *in vacuo* over $CaCl_2$ before analysis. For the determination of ester sulphate, samples were hydrolysed under reflux with 4N-HCl for 8 hr. The hydrolysates were concentrated to dryness *in vacuo* over KOH at 50°, the residue was dissolved in water and inorganic sulphate estimated by the method of Lloyd (1959*c*). Nitrogen was determined with the micro-Kjeldahl apparatus (Markham, 1942) and potassium by the flame photometer.

EXPERIMENTAL AND RESULTS

Preparation of monosaccharide sulphates by the pyridine–sulphur trioxide procedure. Table 1 records the average yields of crude monosaccharide sulphates obtained as the potassium salts after the sulphation of glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine as described earlier. Paper-chromatographic analysis of the reaction products indicated the presence of three components in each case, two containing ester sulphate and a third identical in chromatographic mobility with the parent compound (Table 1).

The sulphation procedure described differs from that of Duff (1949) and Dodgson & Spencer (1954, 1957) in several respects. As preliminary modifications the concentration of pyridine–sulphur trioxide reagent was increased and the reaction was performed in the presence of Drierite, resulting in an average increase in yield of 27% (by wt.) for crude potassium glucose sulphate. To compare the relative concentrations of the components, samples of crude glucose sulphate, prepared by the original

Table 1. *Average yields of crude monosaccharide sulphate esters obtained by the pyridine–sulphur trioxide procedure*

Chromatographic mobilities (R_G) are related to that of glucose, which is taken as unity.

Ester	Yield of crude potassium salt (g./10 g. of starting material)	R_G of components		
		Parent compound	Ester sulphates	
			Di-	Mono-
Glucose sulphate	8·2	1·0	0·66	0·42
Galactose sulphate	8·9	0·95	0·59	0·42
N-Acetylglucosamine sulphate	6·8	1·25	0·76	0·54
N-Acetylgalactosamine sulphate	6·5	1·15	0·71	0·51

and modified procedures, were chromatographed, and the separate components were eluted and estimated by the anthrone procedure. The concentration of the monosulphate component prepared by the modified method corresponded to a 16% increase in yield, as compared with the concentration of this component in mixtures obtained by the earlier methods.

Dodgson & Spencer (1954) reported that samples of potassium glucose sulphate prepared according to Duff (1949) exhibited an absorption peak in the ultraviolet at the wavelength of maximum absorption of pyridine in water. This was presumed to be due to traces of a pyridine salt occurring as an intermediate in the preparation of glucose sulphate. The concentration of this component was substantially decreased after prolonged treatment with aqueous $BaCO_3$, but it was completely removed only after repeated recrystallization of the brucine salt of the ester. In the present method removal of this pyridine complex, as indicated by the disappearance of the ultraviolet absorption, was achieved by stirring the acid aqueous solution of the ester at room temperature and neutralizing with aqueous $Ba(OH)_2$. No change in yield of the glucose monosulphate component of the mixture, determined chromatographically as before, was observed after incorporation of this step.

Separation of sulphated products by paper electrophoresis. Complete separation of monosaccharide monosulphate from the corresponding disulphate and parent monosaccharide could be achieved on Whatman no. 3MM paper in 2 hr. at 400v in 0·1M-ammonium acetate buffer, pH 4·0. The separation was facilitated by applying a hydrodynamic counterflow by raising the level of the buffer in the anode chamber by 3 cm. over that in the cathode chamber. Similar conditions were used to separate 300 mg. of crude potassium glucose sulphate preparation on Whatman no. 17 paper in 4·5 hr. The slowest-moving component isolated from the strip was identified as glucose. The fastest-moving component had R_G 0·42 and was glucose di-*O*-sulphate (Found: ester SO_4^{2-}, 46·3; K, 18·3. Calc. for $C_6H_{10}O_{12}S_2K_2$: ester SO_4^{2-}, 46·1; K, 18·7%). The remaining component had R_G 0·66 and was glucose mono-*O*-sulphate (Found: ester SO_4^{2-}, 33·4; K, 13·2. Calc. for $C_6H_{11}O_9SK$: ester SO_4^{2-}, 32·2; K, 13·1%). Crude preparations of the ester sulphates of galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine could be separated under the same electrophoresis conditions.

Separation of sulphated products by electrophoresis on cellulose columns. The crude salt (0·5 g.), dissolved in 5 ml. of water, was converted into the free acid by passage through a Dowex 50 column (H^+ form; 100–200 mesh; 7 cm. × 1 cm.). The acid eluate and washings were combined (9 ml.) and 2 ml. of cresol red was added as indicator. After draining the buffer (0·1M-ammonium acetate, pH 4·0) in the electrophoresis tube to the level of the cellulose bed, a 5 ml. portion of water was added to the column and allowed to drain in under gravity flow. The sample was then added to the cellulose bed and allowed to drain in as before. Flow through the column was stopped and the space above the cellulose bed washed thoroughly by the successive addition and removal of five 20 ml. portions of water. Finally, a fresh 5 ml. portion of water was added to the column and allowed to drain in slowly. The red indicator band was then displaced slowly down the column to the 30 cm. position by the addition of buffer.

The buffer in the electrode vessels was levelled and current was applied from a stabilized power source to yield a potential difference of 400v, the electrode at the lower end of the electrophoresis tube being made the positive pole in the system. During the first 2 hr. the current rose from 15·0 to 19·5 mA, this period being marked by a distinct sharpening of the indicator zone, which decreased to a width of 0·75 cm. (cf. Porath, 1956). The period of zone-sharpening was also marked by a change in the colour of the indicator band, this change occurring bandwise from the lower edge of the band, until the latter was completely yellow in colour.

When zone-sharpening was complete, the level of buffer in the cathode chamber was lowered by 4 cm. to induce a hydrodynamic counterflow. This

difference in levels was maintained for 28 hr. The electrophoresis was then stopped and the separated zones were displaced with buffer. Eluates corresponding to elution peaks were examined by paper chromatography. The results are represented in Fig. 1.

On a preparative scale 4 g. of the potassium salt of the crude monosaccharide sulphate, dissolved in 15 ml. of water, was converted into the free acid as described above. The sample was applied to the column between two 10 ml. zones of water and displaced to the 30 cm. position with buffer. A potential difference of 500v was applied for 4 hr. The level of buffer in the cathode chamber was then lowered by 4 cm., this difference in levels being maintained throughout the remaining 26 hr. of the separation. Separated zones were then displaced and the components isolated for analysis. Results of typical separations are given below. The components (disulphate, monosulphate and parent compound) are designated according to the order of elution from the column.

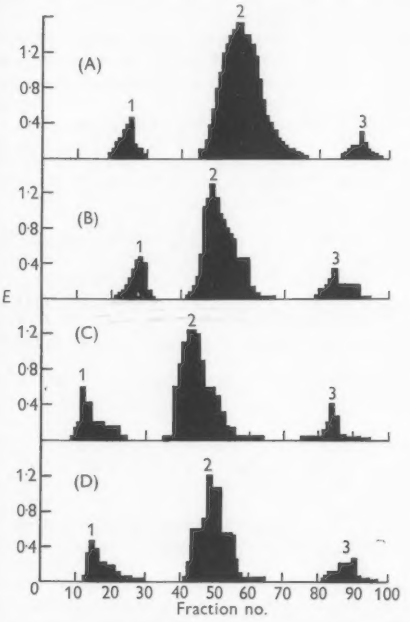

Fig. 1. Zone electrophoresis of crude sulphate esters on cellulose columns. The histograms represent extinctions of fractions in the anthrone procedure (Fairbairn, 1953). (A) Glucose sulphate: component 1, R_G 0·42; component 2, R_G 0·66; component 3, R_G 1·0. (B) Galactose sulphate: component 1, R_G 0·42; component 2, R_G 0·59; component 3, R_G 0·95. (C) *N*-Acetylglucosamine sulphate: component 1, R_G 0·54; component 2, R_G 0·76; component 3, R_G 1·25. (D) *N*-Acetylgalactosamine sulphate; component 1, R_G 0·51; component 2, R_G 0·71; component 3, R_G 1·15.

Glucose sulphate. Component 1, yield 0·8 g., R_G 0·42 (Found: ester SO_4^{2-}, 46·32; K, 18·8. Calc. for $C_6H_{10}O_{12}S_2K_2$: ester SO_4^{2-}, 46·1; K, 18·7%). Component 2, yield 2·3 g., R_G 0·66 (Found: ester SO_4^{2-}, 33·2; K, 12·9. Calc. for $C_6H_{11}O_9SK$: ester SO_4^{2-}, 32·2; K, 13·1%). Component 3, yield 0·22 g., R_G 1·0, was identified as glucose.

Galactose sulphate. Component 1, yield 0·52 g., R_G 0·42 (Found: ester SO_4^{2-}, 46·0; K, 18·9. Calc. for $C_6H_{10}O_{12}S_2K_2$: ester SO_4^{2-}, 46·1; K, 18·7%). Component 2, yield 2·25 g., R_G 0·59 (Found: ester SO_4^{2-}, 32·9; K, 13·3. Calc. for $C_6H_{11}O_9SK$: ester SO_4^{2-}, 32·2; K, 13·1%). Component 3, yield 0·09 g., R_G 0·95, was identified as galactose.

N-Acetylglucosamine sulphate. Component 1, yield 0·72 g., R_G 0·54 (Found: N, 3·9; ester SO_4^{2-}, 41·4; K, 16·8. Calc. for $C_8H_{13}O_{12}NS_2K_2$: N, 3·1; ester SO_4^{2-}, 42·2; K, 17·2%). Component 2, yield 2·1 g., R_G 0·76 (Found: N, 4·4; ester SO_4^{2-}, 28·1; K, 12·1. Calc. for $C_8H_{14}O_9NSK$: N, 4·1; ester SO_4^{2-}, 28·3; K, 11·5%). Component 3, yield 0·12 g., R_G 1·24, was identified as *N*-acetylglucosamine.

N-Acetylgalactosamine sulphate. Component 1, yield 0·12 g., R_G 0·51 (Found: N, 3·7; ester SO_4^{2-}, 42·8; K, 16·8. Calc. for $C_8H_{13}O_{12}NS_2K_2$: N, 3·1; ester SO_4^{2-}, 42·2; K, 17·2%). Component 2, yield 2·2 g., R_G 0·71 (Found: N, 3·8; ester SO_4^{2-}, 28·1; K, 11·2. Calc. for $C_8H_{14}O_9NSK$: N, 4·1; ester SO_4^{2-}, 28·3; K, 11·5%). Component 3, yield 0·19 g., R_G 1·13, was identified as *N*-acetylgalactosamine.

DISCUSSION

The separation of monosaccharide monosulphates from contaminating disulphate and parent compounds has been attempted in several ways. Unchanged glucose has been removed from glucose sulphate preparations by treating the crude sulphation product with yeast, which metabolizes the glucose but has no action on glucose sulphate, or by repeated recrystallization of the monosaccharide sulphate as the brucine salt (Soda, 1933). The former method is time-consuming and the latter (Dodgson & Spencer, 1954) does not result in the complete removal of the contaminating monosaccharide. Neither procedure can be used for the removal of the disulphate compound. Unsuccessful attempts have been made to remove the sulphated impurity by preferential hydrolysis with hydrazine and by selective precipitation of contaminating disulphate with 5-aminoacridine (A. G. Lloyd, unpublished results).

Methods have been reported for the fractionation of crude monosaccharide sulphates by cellulose-

column chromatography (Lloyd, 1959*a*; Turvey & Clancy, 1959) and anion-exchange resins (Turvey & Clancy, 1959). Cellulose-column chromatography is generally unsuitable for the handling of large quantities of material and requires repreparation of the cellulose bed for each separation. Anion-exchange-resin chromatography, although of use in the fractionation of glycoside sulphates, has only limited uses for the purification of reducing monosaccharide sulphates, since degradative changes are catalysed (Dr K. S. Dodgson, personal communication). Zone electrophoresis on cellulose columns has been used successfully for the purification of the adenosine phosphosulphates obtained by the carbodi-imide procedure (Reichard & Ringertz, 1957). The principal advantage of zone electrophoresis for this purpose is that the cellulose bed may be used repeatedly without repreparation. Further, the conditions required for separation are easily reproducible and the application of only moderately high potential differences allow the rapid separation of the components of the mixture. Purification of crude monosaccharide sulphates on a 10 g. scale may be readily achieved with 6 cm. diameter columns.

Evidence about the exact location of the sulphate group in *N*-acetylgalactosamine sulphate is still incomplete. Preliminary examination of the behaviour of the compound in the Elson–Morgan reaction (Lloyd, 1959*a*) suggested that the sulphate group occupies position 6 of the hexosamine molecule. Unsuccessful attempts to prepare triphenylmethyl (trityl) compounds from authentic glucose 6-*O*-sulphate, galactose 6-*O*-sulphate and *N*-acetylglucosamine 6-*O*-sulphate on the one hand, and *N*-acetylgalactosamine monosulphate, on the other hand, may be taken as additional evidence in support of this postulate (A. G. Lloyd, unpublished results). The inability to form trityl compounds from acetylglucopyranose isolated from *Bacillus megaterium* (Duff, Webley & Farmer, 1957; Duff & Webley, 1958) or from cerebron sulphate (Nakayama, 1951) has been taken as a criterion for the establishment of 6-*O*-substitution in these compounds. That this is true has been shown by chemical synthesis of the former (Duff & Farmer, 1958) and methylation studies with the latter (Thannhauser, Fellig & Schmidt, 1955). Further evidence for the location of the sulphate group on position 6 in *N*-acetylgalactosamine monosulphate has been obtained from the infrared spectra of this and related compounds (Lloyd & Dodgson, 1959).

SUMMARY

1. Methods have been developed for the preparation of the crude potassium salts of the sulphates of glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine.

2. Pure potassium salts of glucose 6-*O*-sulphate, galactose 6-*O*-sulphate, *N*-acetylglucosamine 6-*O*-sulphate and *N*-acetylgalactosamine monosulphate have been obtained by zone electrophoresis on cellulose columns.

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Trace Elements and the Synthesis of Vitamin B_{12} by *Streptomyces olivaceus*

By P. K. MAITRA and S. C. ROY
Department of Applied Chemistry, University College of Science and Technology, Calcutta 9, India

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Study of the requirement of trace elements for the streptomycetes has often led to contradictory results (Woodruff, 1947; Spilsbury, 1948; Johnstone & Waksman, 1948). This is mostly due to the difficulty in preparing a truly basal medium and also because such requirements vary with the medium employed (Thornberry & Anderson, 1948). The streptomycete *Streptomyces olivaceus* is one of the few micro-organisms producing significant amounts of vitamin B_{12} (Hall, Benedict, Wiesen, Smith & Jackson, 1953; Hester & Ward, 1954; Pfeifer, Vojnovich & Heger, 1954). It was therefore thought pertinent to make a comparative study of the effect of supplementation of a synthetic basal medium for *S. olivaceus* with various trace elements, singly or in combination, on the growth of mycelia and the synthesis of the vitamin.

EXPERIMENTAL

Organism. The organism used throughout was *Streptomyces olivaceus* NRRL B-1125, kindly provided by Dr C. W. Hesseltine of the Northern Utilization Research and Development Division, Peoria, U.S.A. Details about its maintenance have been reported earlier (Maitra & Roy, 1959*a*).

Media. The medium described by Dulaney & Williams (1953) was modified slightly and contained (per l.): glucose, 20·0 g.; $(NH_4)_2HPO_4$, 7·5 g.; NaCl, 5·0 g.; K_2HPO_4, 1·0 g.; KH_2PO_4, 1·0 g.; $MgSO_4,7H_2O$, 1·0 g.; $CaCO_3$, 1·0 g.; $CaCl_2$, 0·4 g.; $FeSO_4,7H_2O$, 10·0 mg.; $ZnSO_4,7H_2O$, 10·0 mg.; $CoSO_4,7H_2O$, 10·0 mg.

Removal of trace elements. The water used was deionized and distilled twice from an all-glass still. The conductivity determined after boiling for 5 min. was $0{\cdot}72 \times 10^{-6}$ mho at 34° as measured with a Leeds–Northrup conductivity bridge. All the glassware used was either of Pyrex or Jena brand. It was washed first with a detergent, rinsed thoroughly in tap water, soaked for 24 hr. in chromic acid, then washed at least 50 times in tap water, rinsed 10 times with glass-distilled water and finally five times with the deionized double-distilled water and drained dry. Glucose was purified by passing a 20% soln. twice through a column of scrupulously washed Amberlite 1R-120(H^+). The other chemicals used were 'Guaranteed Reagents' from E. Merck and Co. Calcium carbonate was washed with stirring and subsequent settling with 20 changes of deionized double-distilled water. The paste was finally dried in a vacuum desiccator. The sulphates of zinc, cobalt, nickel, manganese, magnesium, chromium and copper were recrystallized once from deionized double-distilled water. The water of crystallization in the recrystallized sample of $Cr_2(SO_4)_3$ was determined, by analysis of a standard solution of the dried sample for H_2SO_4 liberated by passing a portion of it through Amberlite 1R-120(H^+), and was found to be 18. The other salts were used without further purification. To minimize heavy-metal contamination $(NH_4)_2HPO_4$, K_2HPO_4, KH_2PO_4 and NaCl were treated with $CaCO_3$ before use according to Steinberg (1950). In preparing media the element under test was first omitted from and then added to the basal medium in graded doses. Calcium carbonate was added separately to each flask, as was glucose after separate sterilization.

Culture conditions. Except where otherwise stated the inoculum was grown in 100 ml. of the medium described before with no added $CaCO_3$ in 500 ml. Erlenmeyer flasks. The fermentation experiments were conducted in 25 ml. of medium in 100 ml. Erlenmeyer flasks. Incubations were carried out on a rotary shaker (120 rev./min.) at room temperature (about 30°), the periods of inoculum preparation being 96 hr. and that of fermentation proper being 120 hr. Inoculation into the inoculum medium was done with a platinum wire with a pin-point of the stock culture and, after the required period, the suspension was centrifuged at 1500 *g* at 0° to separate cells from the liquid broth, a further 10 washes being made with 150-ml. portions of sterile 0·85% KCl. A weighed portion of the sedimented mycelia was suspended in 0·85% KCl and 0·1 ml. of this suspension (equivalent approximately to 1–2 μg. of N) was used to inoculate each flask containing 25 ml. of the medium.

Analytical procedures. The amounts of mycelia and the vitamin B_{12} produced were determined by methods described by Maitra & Roy (1959*b*). The fermented broth was heated on a water bath for 15 min. at pH 5·5 and the liberated vitamin was estimated in the supernatant with *Escherichia coli* 113-3, by the filter-paper-disk assay. Duplicate flasks were used for each concentration of the element under test. The ash of *S. olivaceus* was obtained by incinerating in a porcelain crucible to 600° a sample of scrupulously washed cells used for the above-mentioned inoculations.

RESULTS

Effect of iron, zinc and cobalt on growth and synthesis of vitamin B_{12} by Streptomyces olivaceus. Since the medium used by Dulaney & Williams (1953) contained iron, zinc and cobalt ions each at approximately 2 p.p.m., the experiments with these ions were designed to vary the doses of the element under test and to keep those of the other

two fixed at 2 p.p.m. The results shown in Fig. 1 indicate that iron and zinc are both essential for synthesis of vitamin B_{12} but added cobalt, on the contrary, is apparently not required; this was observed in a number of similar experiments. This is presumably due to traces of cobalt being present in some component(s) of the medium, for the addition of even 0·2 p.p.m. of the ion considerably stimulated the process.

Effect of copper, manganese, chromium, molybdenum, nickel and boron on growth and synthesis of vitamin B_{12} by Streptomyces olivaceus. The basal medium to which additions of the test elements were made was as used before, except that the concentrations of iron, zinc and cobalt were lowered respectively to 1·0, 1·0 and 0·2 p.p.m. in both the inoculation and the fermentation media. The results in Figs. 2 and 3 indicate that copper is the most effective element for promotion of growth and of vitamin synthesis whereas nickel is inhibitory to synthesis of vitamin B_{12} at all doses studied.

Interchangeability of magnesium and manganese. Magnesium (100 p.p.m.) is present in the synthetic medium of Dulaney & Williams (1953), whereas manganese is not added. In the present experiment the sum total of concentrations of these two ions was kept at this level, except, of course, where both of them were omitted; in that case, however, the sulphur deficiency was made good by adding Na_2SO_4 to the medium.

The effect of replacing magnesium by manganese is shown in Table 1. The results indicate that although these two metals are mutually replaceable, one or other is necessary for synthesis of vitamin B_{12}; an absolute requirement, however, could not be demonstrated. Furthermore, manganese appears to be more effective than magnesium for both growth and synthesis of the vitamin in *S. olivaceus*.

Effect of adding trace elements to deficient medium. The effect of ions added alone or in combination on growth and synthesis of vitamin B_{12} in *S. olivaceus* was examined in a medium deficient in trace elements. The fermentations were carried out in the modified medium of Dulaney & Williams (1953), from which iron, zinc and cobalt were omitted. The inoculum, however, was grown as before on a medium containing 1·0, 1·0 and 0·2 p.p.m. of iron, zinc and cobalt respectively. One of the sets of flasks contained ash materials from the mycelia of *S. olivaceus* (comprising 7·84 % of the dry weight) in place of added trace elements. As the growth rate was very slow, fermentations for this set of experiments were conducted for 180 hr. None of the elements studied when added alone brought about any substantial increase of cell synthesis over that in the unsupplemented medium. Of the various combinations, iron–zinc–cobalt gave the greatest growth and synthesis of vitamin B_{12}; iron–zinc was less effective (Table 2).

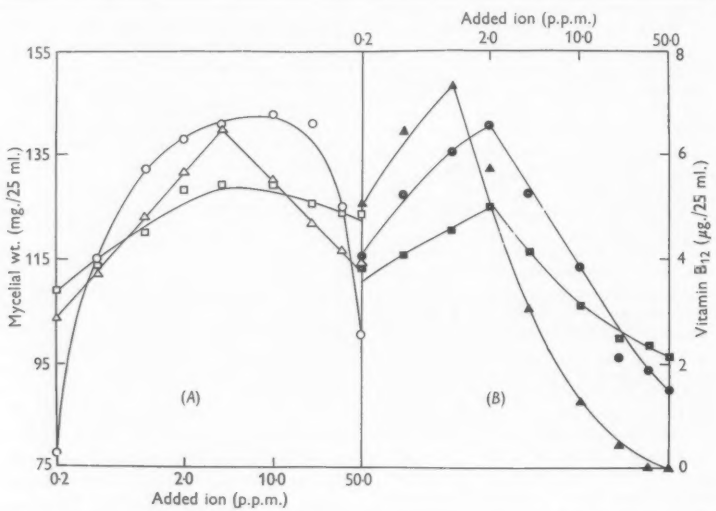

Fig. 1. Effect of iron, zinc and cobalt on mycelial weight and synthesis of vitamin B_{12} in *S. olivaceus*. Representations on the abscissa are in the logarithmic scale. (*A*) Mycelial weight: ○, iron; △, zinc; □, cobalt. The zero levels without addition of ions were 41·8, 57·9 and 95·0 mg./25 ml. respectively. (*B*) Vitamin B_{12} synthesized: ●, iron; ▲, zinc; ■, cobalt. The zero levels without addition of ions were 0, 0 and 1·25 μg./25 ml. respectively.

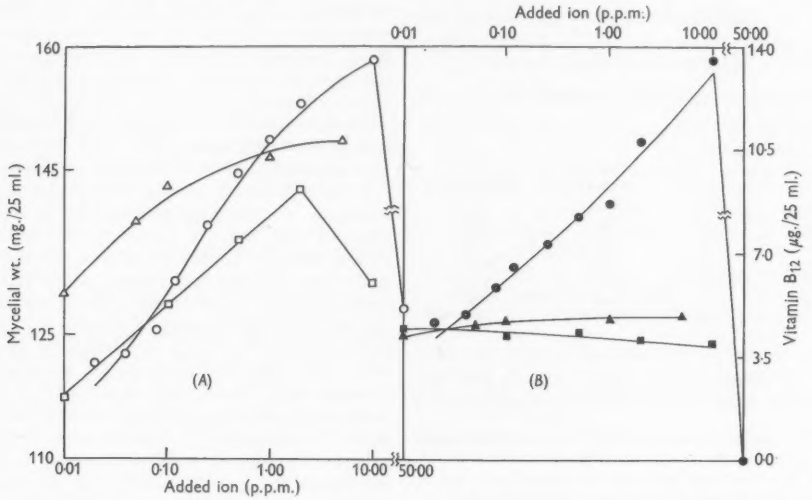

Fig. 2. Effect of copper, manganese and chromium on mycelial weight and synthesis of vitamin B_{12} in *S. olivaceus*. Representations on the abscissa are in the logarithmic scale. (*A*) Mycelial weight: ○, copper; △, manganese; □, chromium. The zero levels without addition of ions were 112·3, 117·4 and 115·9 mg./25 ml. respectively. (*B*) Vitamin B_{12} synthesized: ●, copper; ▲, manganese; ■, chromium. The zero levels without addition of ions were 4·05, 4·15 and 4·20 μg./25 ml. respectively.

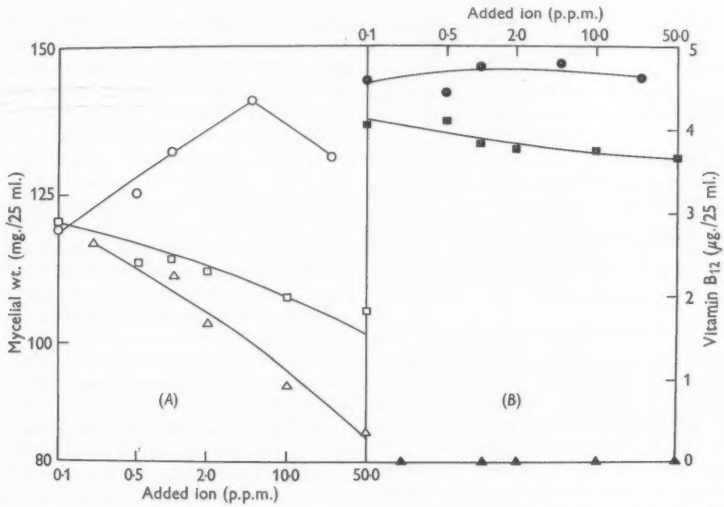

Fig. 3. Effect of molybdenum, nickel and boron on mycelial weight and synthesis of vitamin B_{12} in *S. olivaceus*. Representations on the abscissa are in the logarithmic scale. (*A*) Mycelial weight: ○, molybdenum; △, nickel; □, boron. The zero levels without addition of ions were 117·4, 112·3 and 115·9 mg./25 ml. respectively. (*B*) Vitamin B_{12} synthesized: ●, molybdenum; ▲, nickel; ■, boron. The zero levels without addition of ions were 4·15, 4·05 and 4·20 μg./25 ml. respectively.

Table 1. *Interchangeability of magnesium and manganese in the medium for growth and synthesis of vitamin B_{12} by* Streptomyces olivaceus

Results refer to values for 25 ml. of the medium (details are as given in the text).

Concn. of metals (p.p.m.)		Dry wt. of mycelia	Vitamin B_{12}
Mg	Mn	(mg.)	(μg.)
0	0	90·5	2·25
100	0	118·3	4·25
75	25	146·5	4·90
50	50	144·0	4·75
25	75	149·7	4·80
0	100	149·0	5·00

Table 2. *Effect of trace elements on growth and synthesis of vitamin B_{12} by* Streptomyces olivaceus

Results refer to values for 25 ml. of the medium (details are as given in the text). Incubation was for 180 hr. Figures in parentheses indicate concentration in p.p.m. 'All' indicates the following mixture: Fe (1·0), Zn (1·0), Co (0·2), Cu (1·0), Mo (0·5), Mn (0·1), B (0·1), Cr (0·1) and Ni (0·2).

Additions	Dry wt. of mycelia (mg.)	Vitamin B_{12} (μg.)
None	47·6	0
Fe (1·0)	52·3	Trace
Zn (1·0)	55·6	Trace
Co (0·2)	49·3	0
Cu (1·0)	50·2	0
Mo (0·5)	48·5	0
Mn (0·1)	47·4	0
B (0·1)	43·4	0
Cr (0·1)	46·1	0
Ni (0·2)	36·2	0
Fe (1·0), Zn (1·0)	84·6	1·05
Fe (1·0), Co (0·2)	53·9	0
Zn (1·0), Co (0·2)	45·6	0
Zn (1·0), Ni (0·2)	42·6	0
Fe (1·0), Zn (1·0), Co (0·2)	120·0	3·85
Ni (0·2), Zn (1·0), Co (0·2)	40·8	0
'All'	136·7	0
Ash of *S. olivaceus* mycelium (5·0)	56·2	1·00

DISCUSSION

Heim & Lechevalier (1956) have studied the effect of certain selected ions on the growth of several strains of streptomycetes. Hall *et al.* (1953) studied the requirement of *S. olivaceus* for copper and cobalt in synthesis of vitamin B_{12} in a complex medium and consequently the interpretation of their results is difficult. In the present investigation a synthetic medium has been used to study both growth and synthesis of vitamin B_{12} by *S. olivaceus*. Iron and zinc appear to be necessary for both the processes. Goodwin & McEvoy (1959) have shown that the concentration of iron is critical for flavinogenesis by *Candida flareri*, synthesis being sharply depressed by increasing concentrations of the element. Of a number of trace elements concerned in flavinogenesis, zinc may also be important (Schopfer & Knüsel, 1956). Both in flavinogenesis and synthesis of vitamin B_{12} at least one feature is common, i.e. the formation of nitrogenous ring compounds, and both iron and zinc appear to be involved in the process.

Copper promotes both growth and vitamin B_{12} synthesis in the synthetic medium over a wide range of concentration. Iodice, Richert & Schulman (1958) consider copper to be a component of δ-aminolaevulic acid dehydrase, an enzyme that might possibly be involved in the biosynthesis of the porphyrin-like moiety of vitamin B_{12} (Corcoran & Shemin, 1957). Boron is detrimental to growth, although it does not much inhibit the synthesis of vitamin B_{12}. Such disproportionality between vitamin B_{12} and cell syntheses was reported also by Kurz & Nielsen (1957) working with *Streptomyces griseus*. Chromium, molybdenum and manganese accelerate the growth a little but are practically without effect on synthesis of the vitamin. The inhibitory effect of nickel on both the processes is also significant. The effect of nickel on the decreased synthesis of vitamin B_{12} by *S. olivaceus* was not an artifact of suppressed growth of the assay organism *E. coli* 113–3, since the latter could grow equally well at a concentration of nickel that could inhibit the synthesis of vitamin B_{12} by *S. olivaceus*.

Whether the effect of these trace elements on growth and synthesis of vitamin B_{12} by *S. olivaceus* is direct or indirect is also an important point. Some of the trace elements studied here are either activators or constituents of many enzyme systems (Neilands & Stumpf, 1958) and may be concerned in the growth and synthesis of vitamin B_{12} by *S. olivaceus* in this way.

SUMMARY

1. A glucose–inorganic salt medium has been rendered as free as possible from extraneous metal ions, and the effect of some trace elements on growth and synthesis of vitamin B_{12} by *Streptomyces olivaceus* has been studied.

2. In such a system iron and zinc are essential for synthesis of vitamin B_{12} and cobalt at low levels is stimulatory.

3. Copper favours both growth and synthesis of vitamin B_{12} up to 10 p.p.m. Molybdenum, chromium and manganese are favourable for growth up to 5, 2 and 100 p.p.m. respectively but have no significant effect on the synthesis of the vitamin.

4. Nickel is inhibitory to both growth and synthesis of the vitamin at concentrations as low as 0·2 p.p.m., whereas boron inhibits only the growth.

5. Magnesium can be totally replaced by manganese in the medium.

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The Effect of Insulin *in vitro* on the Accumulation of Amino Acids by Isolated Rat Diaphragm

BY K. L. MANCHESTER AND F. G. YOUNG
Department of Biochemistry, University of Cambridge

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It is now well established that insulin *in vitro* enhances the incorporation of labelled amino acids into the protein of isolated rat diaphragm (Sinex, MacMullen & Hastings, 1952; Krahl, 1953; Manchester & Young, 1958*a*; Wool & Krahl, 1959*a*). This effect of insulin, which is not reproduced *in vitro* by any other hormones examined in this respect (Manchester & Young, 1959; Manchester & Young, in preparation), is not dependent upon or affected by the presence of glucose in the medium, and addition of glucose alone under most conditions has no observable effect on amino acid incorporation.

Since insulin accelerates the rate of entry of glucose and of a variety of non-utilizable sugars into the muscle cell (Levine & Goldstein, 1955; Park, Bornstein & Post, 1955; Helmreich & Cori, 1957; Park, Johnson, Wright & Batsel, 1957), it seemed to us possible that the stimulation by insulin of incorporation of labelled amino acids into the protein of diaphragm might be a consequence of an enhancement by this hormone of the rate of entry of amino acids into the tissue cells. Evidence in support of such a view was found by Kipnis & Noall (1958), when they showed that insulin *in vitro* enhanced the rate of accumulation, and the maximum accumulation effected, of a non-utilizable amino acid (α-amino*iso*butyric acid) by the isolated intact diaphragm preparation of Kipnis & Cori (1957). We have reinvestigated the effect of insulin on the accumulation of α-amino*iso*butyric acid by both intact and ordinary 'cut' diaphragm preparations, and have also studied the effect of insulin on the accumulation of a number of utilizable, naturally occurring amino acids.

METHODS

Radioactive materials. Radioactive amino acids were obtained from The Radiochemical Centre, Amersham, Bucks. Glycine and α-amino*iso*butyric acid had ^{14}C in the carboxyl position; alanine, leucine, phenylalanine, arginine, lysine, aspartic acid and glutamic acid were all the L-isomers and uniformly (U) labelled with ^{14}C. The concentrations at which the amino acids were added to the medium are indicated in the tables and figures. In the experiments with intact diaphragm the amount of radioactivity added to the medium (irrespective of the quantity of amino acid) was 0·33 μc/ml.; in the experiments with cut diaphragm the amount of radioactivity added to the medium was 0·4 μc/ml.

[U-^{14}C]Ornithine was made from [U-^{14}C]arginine by a modification of the method of Mohamed & Greenberg (1945) with arginase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). It was purified by paper electrophoresis.

Medium. The balanced salt bicarbonate buffer of Gey & Gey (1936) was used throughout, gassed with O_2+CO_2 (95:5, v/v) or N_2+CO_2 (95:5, v/v) (pH 7·4 at 37°), with or without addition of glucose (2·5 mg./ml.). Ox insulin (Boots Pure Drug Co. Ltd.), six times recrystallized, was dissolved in 3·3 mN-HCl to a concentration of 20 units/ml. and diluted with Geys' buffer to 0·1 unit/ml. on the day of the experiment.

Rats. Diaphragms were removed from female albino Wistar-strain rats about 100–120 g. in weight. The rats had been bred in the Department and fed on a standard laboratory diet (MRC-41, Bruce & Parkes, 1949). They were deprived of food about 16 hr. before death, but had free access to water at all times.

Removal and incubation of diaphragm. Experiments with sugars (Kipnis & Cori, 1957; Randle & Smith, 1958) have suggested that the usual isolated hemidiaphragm, because of its cut edges, is not a satisfactory preparation for the measurement of a sugar 'space', and that the intact-diaphragm preparation, introduced by Kipnis & Cori (1957), in which the diaphragm remains uncut within the rib case, is more suitable in this respect. In the present investigation we have used both preparations, both being removed as soon as practicable after death and washed for about 10 min. at 10° in freshly gassed buffer containing no glucose, insulin or amino acid. Intact diaphragms were placed in 50 ml. conical flasks containing 5 ml. of medium. They were gassed, sealed and incubated at 37° with continuous shaking. In some experiments, involving the construction of a curve relating time and effect, hemidiaphragms were incubated individually in small conical flasks containing 1 ml. of medium and gassed and sealed before incubation. In other experiments involving only one period of time, four 50 ml. conical flasks were set up, each containing 6 ml. of medium. The medium in the first flask contained no insulin or glucose; the second, glucose but no insulin; the third, insulin but no glucose; and the fourth, both glucose and insulin. Hemidiaphragms were removed from 12 rats, systematically mixed so that hemidiaphragms from the same rat were never contained in the same flask, and six were added to each flask, the flasks being gassed and sealed as before. In the experiments in which the time allowed for the accumulation of labelled amino acids was 5–15 min. the labelled amino acid was added to the medium after incubation of the diaphragm had begun in order that the length of time for which the diaphragm was in contact with labelled amino acid at 37° might be precisely fixed.

Measurement of the accumulation of soluble radioactive material. After incubation the diaphragm tissue from intact diaphragms was dissected away from the rib cage. Diaphragm tissue so obtained, and also hemidiaphragm (cut tissue), was carefully blotted, weighed and placed in 2 ml. of water at 100° for 5 min. After centrifuging, a 1 ml. sample of the soluble extract was transferred to a 2 cm. diam. aluminium planchet that had been painted with pale gold lacquer to bind the residue, and the extract was evaporated to dryness under an infrared lamp and counted beneath a thin end-window counter. A sample (0·2 ml.) of the medium after incubation was diluted to 5 ml. with water and 1 ml. of the diluted material was plated, dried and counted in a like manner. Counts/min. ranged between 500 and 3000. The number of counts/unit wt. of diaphragm water was calculated on the assumption that the diaphragm tissue contains 75% of water, and this figure was divided by the number of counts remaining in the incubation medium to give a concentration ratio for tissue/medium.

Although extracts of the tissue and medium prepared in this fashion contain a little protein into which amino acids may have been incorporated, calculation shows that the contribution of this protein to the amount of radioactivity measured in these extracts is negligible. In preliminary experiments with glycine, phenylalanine and lysine it was found that more than 95% of the radioactivity present in the extract of the tissue was recoverable by paper chromatography in the fraction corresponding to the amino acid added to the medium. Kipnis & Noall (1958) reported a similar finding for α-amino*iso*butyric acid.

Measurement of incorporation. Samples of protein for measurement of their content of radioactivity were prepared as described by Manchester & Young (1958*a*). Incubation at 12° reduced incorporation to a few per cent of the amount at 37°. Negligible incorporation of α-amino*iso*-[^{14}C]butyric acid into protein was observed. Small but measurable incorporation from [^{14}C]ornithine into protein was found, but whether this was a property of the ornithine or an artifact due to contamination of the ornithine by arginine was not certain.

Design of experiments. Experiments on the effect of insulin on the accumulation of free radioactive amino acids in diaphragm have been carried out in two ways. First, the accumulation at a number of different time intervals has been measured; this, of practical necessity, has been based on single observations at multiple times. Secondly, observations of the accumulation during a single period of incubation have been made. The latter procedure has the advantage that statistical analysis can be applied to the figures obtained; the former procedure ensures that one does not start by doing a large number of experiments, designed to provide statistically significant information, at a time period when the insulin effect is less apparent than at others.

Christensen & Streicher (1949) found that for there to be net uptake of amino acid from the medium by isolated rat diaphragm the amino acid must be added to the medium in a concentration greater than that in which it is normally present in blood plasma; for a concentration of amino acid less than that in plasma there was a net outflow of amino acid from the tissue into the medium. Within a physiological range of amino acid concentrations the ratio of the amount of amino acid in the tissue to that in the medium remained constant and did not vary with changes in the external concentration. In our investigation it appeared probable that addition of labelled amino acids to the medium at the lowest practicable concentration, when the net inflow of amino acid in the absence of insulin is at a minimum, would be the conditions under which an effect of insulin on accumulation of amino acid by diaphragm would be most easily demonstrable. We have done experiments of this sort, mostly involving the construction of a curve relating time and effect (e.g. Figs. 1–3), and we have also studied the effect of insulin on the accumulation of amino acids by diaphragm when the amount of amino acid added to the medium is considerably in excess of that found in

plasma (see Tables 1 and 2). Insulin is already known to stimulate incorporation of amino acid into protein of isolated rat diaphragm at all the concentrations of amino acids used in these experiments (compare figures in Manchester & Young, 1958*a*, 1959; Manchester, Randle & Smith, 1958).

RESULTS

Accumulation in vitro *of amino acids by isolated intact and cut rat diaphragm*

A comparison of the figures in Tables 1 and 2 reveals that in 90 min. a greater accumulation of most amino acids is effected by cut diaphragm than by the intact preparation. The reason for this is uncertain. If cut diaphragm lost amino acid through its cut edges lower figures for accumulation by cut diaphragm than for intact would be expected. It is possible that accumulation is initially more rapid with cut diaphragm than with the intact, although similar values would ultimately be attained.

In the majority of our experiments the ratio of the concentration of free labelled amino acid in tissue water to that in the medium was greater than unity, which suggests that a concentration gradient is maintained between the tissue and the medium. The abnormally low ratios for glutamic acid and aspartic acid (Table 1) are probably a consequence of the rapid breakdown and metabolism of these acids in diaphragm tissue *in vitro* (Manchester & Young, 1958*b*). Christensen & Streicher (1949) found a ratio of 3–5 for glycine for isolated diaphragm. Noall & Kipnis (1959) obtained a figure of 7 for the concentration ratio of α-amino*iso*butyric acid between diaphragm and plasma *in vitro*.

Cooling to 12°, which interferes with energy production both by oxidation and by glycolysis, reduced the ratio for phenylalanine and glycine to unity or below, both in the presence and the absence of added glucose (Tables 1 and 2). Anoxia, which interferes with energy production by oxidation but not by glycolysis, reduced the ratio for glycine and arginine nearly to unity in the absence of added glucose, but in the presence of glucose the ratio was significantly higher than in its absence (Table 2).

Effect of insulin in vitro *on accumulation of amino acids by diaphragm*

Addition of insulin to the medium accelerated the rate at which α-amino*iso*[^{14}C]butyric acid entered the intact-diaphragm preparation (Fig. 1). The time curve for the accumulation of this amino acid rose steeply over the first hour of incubation, but was still rising, though slowly, after 3 hr. At the end of 90 min. of incubation insulin had significantly increased the concentration ratio both with the intact-diaphragm preparation and the cut preparation (Tables 1 and 2). Although the accumulation was greater for the cut than for the intact preparation, the effect of the added insulin, on a percentage basis, was about the same (240 and 220% respectively). Addition of glucose in no case significantly affected the accumulation of α-amino*iso*butyric acid (Tables 1 and 2).

As was found with α-amino*iso*butyric acid, insulin raised the accumulation of [^{14}C]glycine by diaphragm, intact (Table 1) or cut (Table 2), both in the presence and the absence of added glucose in the medium. The insulin effect, however, was not as striking as with α-amino*iso*butyric acid. A possible effect of insulin was seen in the time curves for glycine (Figs. 1 and 2) at a majority of the time intervals between 30 min. and 3 hr., but the significance of the differences is doubtful. Addition of glucose had no consistent effect on glycine accumulation.

Addition of insulin had no stimulating effect on the accumulation, after 90 min., of [^{14}C]alanine, [^{14}C]phenylalanine, [^{14}C]lysine, [^{14}C]glutamic acid and [^{14}C]aspartic acid by the intact diaphragm (Table 1) or of [^{14}C]leucine, [^{14}C]phenylalanine and [^{14}C]lysine by the cut preparation (Table 2). Similar results are apparent from the time curves for alanine and phenylalanine (Fig. 1) and for phenylalanine, lysine, glutamic acid and ornithine (Fig. 2).

One of the difficulties of showing an effect of insulin on glucose transport is the rapid rate at which glucose is utilized once it has entered the tissue. Park *et al.* (1955) found that by incubating isolated rat diaphragm at 12° utilization of glucose was sufficiently retarded to allow free glucose to accumulate within the diaphragm. Under these conditions they found that insulin accelerated the entry of glucose. The possibility that insulin, by direct or indirect action, so raised the rate of transfer of labelled amino acids from the cell pool into protein that the observation of an effect of insulin on the accumulation of amino acids was masked, seems most unlikely, since the amount of free radioactivity present in the tissue as free amino acid at any time is vastly in excess of the amount being incorporated into protein. Nevertheless, we have studied the effect of insulin on the accumulation in diaphragm of amino acids under conditions where the incorporation of these amino acids into protein is reduced to negligible proportions. Incorporation has been inhibited (*a*) by cooling to 12°, which greatly slows down enzymic reactions, (*b*) by anoxia, which interferes with the production of energy-rich phosphate needed for incorporation (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Manchester & Young, 1959). Under neither of these conditions did insulin have an observable effect on the accumulation of glycine, phenylalanine or arginine (Tables 1 and 2).

Table 1. *Effect of insulin and glucose* in vitro *on the accumulation of labelled amino acids by the isolated intact rat-diaphragm preparation*

Insulin was used at 0·1 unit/ml. and glucose at 2·5 mg./ml. Each figure is the average of six observations. In each instance the mean result is given ±S.E.M. Length of incubation was 90 min. in every case except for the experiment marked with an asterisk (*), where it was 120 min. N.S., not significant.

Amino acid added to the medium	Concn. of amino acid (μmole/ml.)	Ratio: Concn. of free labelled amino acid in tissue water / Concn. of free labelled amino acid in incubation medium				Significance of differences
		No insulin; no glucose (*a*)	No insulin; glucose (*b*)	Insulin; no glucose (*c*)	Insulin and glucose (*d*)	
Concn. of added amino acid greater than that in rat plasma						
α-Amino*iso*[1-^{14}C]butyric acid	0·15	0·77±0·026	0·74±0·042	1·63±0·09	1·67±0·13	(*c*) – (*a*) $P < 0·001$, (*d*) – (*b*) $P < 0·001$
[1-^{14}C]Glycine	1·0	1·19±0·03	1·36±0·07	2·09±0·04	1·70±0·07	(*c*) – (*a*) $P < 0·001$, (*d*) – (*b*) $P < 0·01$, (*b*) – (*a*) P 0·05, (*c*) – (*d*) P 0·001
L-[U-^{14}C]Alanine	1·0	1·24±0·049	1·33±0·024	1·38±0·086	1·36±0·072	N.S.
L-[U-^{14}C]Phenylalanine	1·0	1·33±0·054	1·32±0·040	1·26±0·029	1·07±0·085	(*b*) – (*d*) $P < 0·05$
L-[U-^{14}C]Lysine	1·0	1·14±0·048	1·14±0·086	0·90±0·055	1·01±0·051	(*a*) – (*c*) $P < 0·01$
L-[U-^{14}C]Glutamic acid	1·0	0·71±0·02	0·75±0·04	0·74±0·04	0·70±0·02	N.S.
L-[U-^{14}C]Aspartic acid	1·0	0·61±0·026	0·59±0·028	0·64±0·028	0·58±0·020	N.S.
Concn. of added amino acid less than that in rat plasma						
[1-^{14}C]Glycine*	0·1	—	1·54±0·09	—	2·47±0·25	(*d*) – (*b*) $P < 0·01$
L-[U-^{14}C]Lysine	0·05	—	1·04±0·036	—	1·08±0·05	N.S.
Incubation carried out at 12°						
L-[U-^{14}C]Phenylalanine	1·0	1·04±0·037	0·95±0·037	1·03±0·033	1·08±0·14	N.S.

Table 2. *Effect of insulin and glucose* in vitro *on the accumulation of labelled amino acids by the isolated cut rat-diaphragm preparation*

Insulin was used at 0·1 unit/ml. and glucose at 2·5 mg./ml. Each figure is the average of six observations. In each instance the mean result is given ±S.E.M. The length of incubation was in every case 90 min.

Amino acid added to the medium	Concn. of amino acid (μmole/ml.)	Ratio: Concn. of free labelled amino acid in tissue water / Concn. of free labelled amino acid in incubation medium				Significance of differences
		No insulin; no glucose (*a*)	No insulin; glucose (*b*)	Insulin; no glucose (*c*)	Insulin and glucose (*d*)	
		Incubation at 37°; O_2+CO_2				
α-Amino*iso*[1-^{14}C]butyric acid	0·22	2·03±0·10	2·40±0·17	5·35±0·28	5·30±0·33	(*c*) − (*a*) $P < 0·001$, (*d*) − (*b*) $P < 0·001$
[1-^{14}C]Glycine	1·0	3·57±0·16	3·63±0·12	4·36±0·35	4·44±0·39	(*c*) − (*a*) $P < 0·1$, (*d*) − (*b*) $P < 0·1$
L-[U-^{14}C]Leucine	1·0	3·69±0·23	3·64±0·18	4·02±0·18	3·32±0·12	(*c*) − (*d*) $P < 0·01$
L-[U-^{14}C]Phenylalanine	1·0	1·67±0·31	1·61±0·046	1·54±0·024	1·53±0·027	N.S.
L-[U-^{14}C]Lysine	1·0	2·68±0·064	2·47±0·073	2·49±0·098	2·31±0·099	(*a*) − (*b*) $P < 0·05$
		Incubation at 12°; O_2+CO_2				
[1-^{14}C]Glycine	1·0	0·85±0·036	0·89±0·024	0·86±0·033	0·87±0·028	N.S.
		Incubation at 37°; N_2+CO_2				
[1-^{14}C]Glycine	1·0	1·18±0·011	1·49±0·034	1·24±0·019	1·56±0·035	(*b*) − (*a*) $P < 0·001$, (*d*) − (*c*) $P < 0·001$
L-[U-^{14}C]Arginine	1·0	1·18±0·021	1·33±0·036	1·23±0·013	1·51±0·034	(*b*) − (*a*) $P < 0·01$, (*d*) − (*c*) $P < 0·001$, (*d*) − (*b*) $P < 0·01$

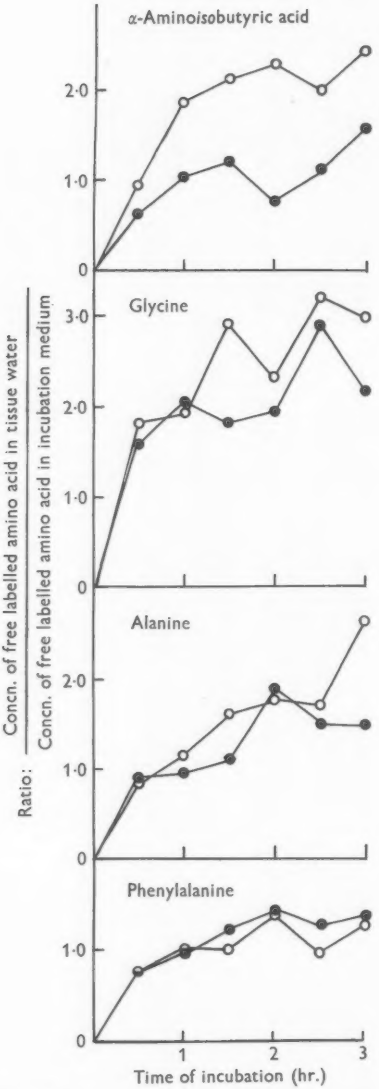
α-Aminoisobutyric acid
Glycine
Alanine
Phenylalanine
Ratio: Concn. of free labelled amino acid in tissue water / Concn. of free labelled amino acid in incubation medium
Time of incubation (hr.)

Fig. 1. Effect of insulin (0·1 unit/ml.) *in vitro* on the rate of accumulation of free labelled amino acids by the isolated intact rat-diaphragm preparation. ●, No insulin added; ○, insulin added. Each point represents a single observation. No glucose was added to the medium. The concentrations (μmole/ml.) of amino acids added to the medium were: α-amino*iso*butyric acid, 0·1; glycine, 0·1; L-alanine, 0·06; L-phenylalanine, 0·035.

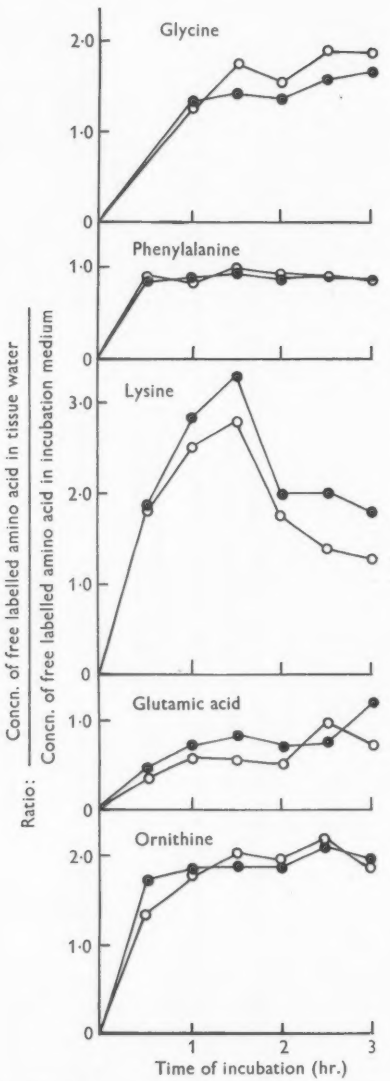
Glycine
Phenylalanine
Lysine
Glutamic acid
Ornithine
Ratio: Concn. of free labelled amino acid in tissue water / Concn. of free labelled amino acid in incubation medium
Time of incubation (hr.)

Fig. 2. Effect of insulin (0·1 unit/ml.) *in vitro* on the rate of accumulation of free labelled amino acids by the isolated cut rat-diaphragm preparation. ●, No insulin added; ○, insulin added. Each point represents a single observation. Glucose (2·5 mg./ml.) was added to the medium. The concentrations (μmole/ml.) of amino acids added to the medium were: glycine, 0·1; L-phenylalanine, 0·035; L-lysine, 0·055; L-glutamic acid, 0·08; L-ornithine, 0·1.

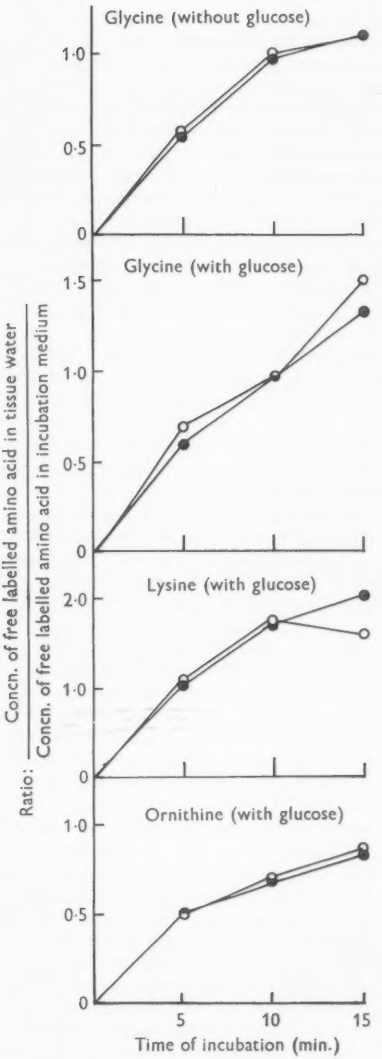
Glycine (without glucose)
Glycine (with glucose)
Lysine (with glucose)
Ornithine (with glucose)
Ratio: Concn. of free labelled amino acid in tissue water / Concn. of free labelled amino acid in incubation medium
Time of incubation (min.)

Fig. 3. Effect of insulin (0·1 unit/ml.) *in vitro* on the rate of accumulation of free labelled amino acids by the isolated cut rat-diaphragm preparation. ●, No insulin added; ○, insulin added. With glycine and lysine each point represents a single observation. Each point for ornithine is the average of two observations. The concentrations (μmole/ml.) of amino acids added to the medium were: glycine, 0·1; L-lysine, 0·055; L-ornithine, 0·1.

Since the time curves described so far suggest that the attainment of equilibrium between the free amino acid in the tissue and that in the medium takes place rapidly, it seemed possible that the effect of insulin on this process, if in fact there is one, might be more apparent in the early stages of incubation than at the later time periods studied; insulin, although not affecting the final equilibrium, might accelerate the rate at which this equilibrium was reached. We have therefore looked for an effect of insulin on the accumulation of labelled amino acids by diaphragm during the first few minutes of incubation. No effect of insulin on the rate of accumulation of glycine, lysine or ornithine could be detected in periods of 5–15 min. (Fig. 3).

DISCUSSION

The fact that ratios approaching unity were obtained at 12° and under anoxic conditions in the absence of glucose suggests that amino acids are able to penetrate approximately the whole of the tissue water of isolated rat diaphragm in the absence of an active process of concentration (cf. measurements for xylose by Kipnis & Cori, 1957, and by Randle & Smith, 1958). Nevertheless the concentration of amino acid may not be uniform throughout the compartments of the tissue, and in particular may be different in the extracellular and intracellular fluids. If the extracellular fluid always contains the same concentration as the medium, a mean concentration ratio for the whole tissue which is above unity may be seriously less than the true ratio for the intracellular fluid and medium.

The finding of Kipnis & Noall (1958) that insulin stimulates the accumulation of α-amino*iso*butyric acid by the intact-diaphragm preparation has been confirmed; we further find that the insulin effect is equally apparent when cut diaphragm is used. We have also found that insulin stimulates accumulation of glycine by diaphragm, both cut and intact, but with none of the other eight naturally occurring amino acids investigated have we found any evidence to suggest that insulin stimulates their accumulation by either cut or intact diaphragm. Although our results do not preclude the possibility that insulin stimulates the transfer of amino acids into some small fraction of the cell volume, e.g. microsomes or mitochondria, an effect which would be too small to be observable in this type of experiment, we are forced to conclude that with the possible exception of glycine we have failed to obtain evidence that the stimulation of the incorporation of labelled amino acids into protein follows an enhancement by insulin of the accumulation of these amino acids in the intracellular fluid of the tissue. Such a conclusion is in accord with two recent observations. Wool & Krahl (1959*b*)

find that insulin *in vitro* stimulates the incorporation into diaphragm protein of labelled amino acids accumulated *in vivo* before removal of the diaphragm; that is, under conditions where the bulk of the free amino acid is probably intracellular and where the extracellular concentration of labelled amino acid has been reduced during the presoaking of the diaphragm. Furthermore, Manchester & Krahl (1959) find that insulin stimulates the incorporation into diaphragm protein of ^{14}C from a number of ^{14}C-labelled carboxylic acids and from $^{14}CO_2$, results which suggest that insulin enhances the incorporation of amino acids, formed presumably intracellularly, from various precursors, under conditions where transport of amino acids from extracellular to intracellular fluid is not involved. An effect of insulin on the accumulation of carboxylic acids by diaphragm was looked for but not found. In contrast with the concentration ratios for most amino acids studied those for many carboxylic acids are less than unity (Manchester & Krahl, 1959).

If indeed insulin has no effect on the accumulation of most amino acids by diaphragm, why should α-amino*iso*butyric acid and glycine behave exceptionally in this respect? Structurally, α-amino-*iso*butyric acid differs from all the naturally occurring amino acids since its α-carbon atom carries no hydrogen atom. Such a structure makes the α-carbon a potential tertiary radical, the α-carbon of the other amino acids in proteins, with the exception of glycine, being potential secondary radicals. The figures in the time curves suggest that the rate of attainment of the maximum level of accumulation of α-amino*iso*butyric acid may be slower than for naturally occurring amino acids. It is conceivable that the presence of a second group on the α-carbon in some way, perhaps by steric hindrance, retards the rate of entry of α-amino*iso*-butyric acid into the muscle cell and at the same time makes it more susceptible to an effect of insulin in easing its entry. Incidentally, both α-amino*iso*butyric acid and glycine, the two amino acids whose accumulation by diaphragm is stimulated by insulin, differ from other naturally occurring amino acids in that they are not optically active. The negative results with ornithine are of particular interest in that ornithine is an amino acid which is believed not to be incorporated into protein (see Methods section), i.e. another non-utilizable amino acid as is α-amino*iso*butyric acid. Whereas the effect of insulin on accumulation of α-amino*iso*butyric acid was very marked there was no observable effect with ornithine.

Kipnis & Noall (1958) observed that addition of glucose in either the presence or the absence of insulin slightly depressed the accumulation of α-amino*iso*butyric acid. This observation we have not confirmed. In our experiments glucose did not enhance accumulation of amino acids, except in the experiments under anoxic conditions with cut diaphragm (Table 2), in which it may have provided energy by glycolytic breakdown. Nor did it appear consistently to compete with and therefore retard the entry of amino acids (Tables 1 and 2). Competition between different sugars (Battaglia & Randle, 1959) and different amino acids (Christensen, Cushing & Streicher, 1949) for entry into the diaphragm-muscle cell has been previously observed. No evidence has been found for the competition of glucose with amino acids for entry into isolated rat diaphragm in our experiments, which suggests that sugars and amino acids are carried into the cell by different transport mechanisms. Since sugars do not appear to be actively concentrated, whereas amino acids are, this non-identity of transport mechanisms is not surprising. It is also in accord with the observation with isolated diaphragm that addition of phlorrhizin at a concentration in which it strongly depresses uptake of glucose and penetration by xylose has no effect on amino acid incorporation into protein (Battaglia, Manchester & Randle, in preparation).

In all our experiments it is the movement and accumulation of label that has been measured, not that of the amino acid itself. It is not possible to deduce from these figures how much unlabelled amino acid is lost by the tissue to the medium during the course of the uptake of labelled amino acid. Nor is it possible to assess from our measurements to what extent entry of labelled amino acid represents net uptake of amino acid by the tissue, what proportion of the label entering the tissue does so through the entry of labelled amino acid in exchange for an equal quantity of unlabelled amino acid of the same species leaving the tissue, or to what extent labelled amino acid enters the tissue as amino acid of another variety leaves (Christensen, Hess & Riggs, 1954). The existence of so many circumstances in which labelled amino acids can accumulate in diaphragm complicates attempts to explain the enhancement by insulin of incorporation of labelled amino acids into protein as a consequence of an effect of insulin on the transport of amino acids into the cell. Since the rate of entry of labelled amino acids from the medium into diaphragm is so rapid, the rate-limiting process, which is presumably accelerated by insulin, may well be elsewhere in the sequence of reactions by which labelled amino acids move from the medium into the protein of diaphragm.

SUMMARY

1. The rate of penetration into isolated rat diaphragm, both ordinary 'cut' preparation and 'intact' preparation, of ^{14}C present in the medium

in certain [^{14}C]amino acids has been investigated, together with the effect of insulin thereon. No important differences between the behaviour of the cut and intact preparations was observed.

2. With alanine, α-amino*iso*butyric acid, glycine, leucine, lysine, ornithine and phenylalanine the ratio of concentration of radioactivity in tissue water to that in the medium exceeded unity. With glutamic acid and aspartic acid the ratio was below unity, probably because of the rapid metabolism of these amino acids in isolated rat diaphragm.

3. The addition of insulin to the medium significantly raised the ratio for glycine and α-amino*iso*butyric acid, but not for the other amino acids under study. The effect of insulin was similar whether or not glucose was added to the medium. It was abolished by anoxia and by cooling the system to 12°.

4. Under anoxic conditions, both in the presence or absence of insulin, the ratio was significantly raised by the addition of glucose to the medium. A similar effect of glucose was not seen under any other conditions studied.

5. No substantial evidence has been obtained to suggest that insulin promotes the incorporation of amino acids into protein in isolated diaphragm by enhancing the rate of entry of amino acids into the cytoplasm of the cells from the medium.

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Phosphoinositides

1. CONFIGURATION OF THE INOSITOL PHOSPHATE IN LIVER PHOSPHATIDYLINOSITOL

BY J. N. HAWTHORNE
Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham 15

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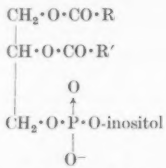
Work from several different Laboratories suggests that the inositol-containing lipid which occurs in liver and heart has structure (I) (Faure & Morelec-Coulon, 1954; Hawthorne, 1955*a*; McKibbin, 1956; Hanahan & Olley, 1958). The L-α-glycerophosphate structure, which might be expected from comparison with phosphatidylcholine, has not yet been rigidly proved. There is also doubt about the point of attachment of the phosphoric acid to the *myo*inositol ring, which is the

subject of this paper. The name 'monophosphoinositide' has been widely used for this compound. As the structure is now known to be analogous to that of phosphatidylcholine, phosphatidylinositol seems a more suitable name.



(I) Phosphatidylinositol (R and R′ are long-chain alkyl residues)

The studies of Brown and his colleagues (e.g. Brown, Hall & Higson, 1958) and the unambiguous synthesis of glycerol 1-(inositol 2-phosphate) (Ellis & Hawthorne, 1959; Davies & Malkin, 1959), enable deductions to be made about the configuration of the inositol phosphate in phosphatidylinositol. These depend on a study of the proportions of the inositol and glycerol phosphates obtained by alkaline hydrolysis of the lipid. The diester from the lipid is referred to as glycerylphosphorylinositol. A preliminary account of some of this work has already appeared (Hawthorne & Hübscher, 1959*b*).

MATERIALS AND METHODS

Analytical methods. These have been described by Hawthorne & Hübscher (1959*a*).

Resin columns. Nalcite SAR (200–400 mesh) proved to be the most suitable resin for the separation of inositol and glycerol phosphates. This was obtained from the National Aluminate Corp., Chicago, 38, and not from the Dow Chemical Company as previously stated. Dowex 2 (×8, 200–400 mesh) is a similar anion-exchanger but was not quite so effective in the separation. This may have been partly due to the higher proportion of fine particles in the Nalcite SAR.

Preparation of phosphatidylinositol. This was obtained from ox liver. A crude ethanol-insoluble phospholipid fraction was first prepared (cf. Hawthorne, 1955*a*). The liver, obtained a few hours after the death of the animal, was minced and then homogenized for 1 min. in a high-speed mixer with chloroform–methanol (2:1, v/v). The volume was then made to 3 l./kg. of fresh liver with the same solvent. After 1 hr. the mixture was filtered at the pump and the residue re-extracted with a further 3 l./kg. of the chloroform–methanol. The upper layers of aqueous methanol were rejected. The combined extracts were then concentrated in a Cyclone evaporator (Quickfit and Quartz Ltd., Stone, Staffs.) at 30° until a viscous oil began to separate (approx. 0·1 vol.). Phospholipids were precipitated by the addition of 5 vol. of acetone to the concentrated extract. The mixture was then stored overnight at 4°. The original precipitate and any further lipid which came down on keeping were dissolved in 500 ml. of chloroform/kg. of fresh liver (subsequent volumes all refer to 1 kg. of fresh liver). The solution was dried over anhydrous Na_2SO_4, filtered and phospholipid was reprecipitated with 3 vol. of acetone.

The sticky, yellowish precipitate was then dissolved in 300 ml. of chloroform and shaken well with 2 vol. of absolute ethanol. The resulting 'cephalin' precipitate was dissolved in 500 ml. of ether and stored at 4° overnight. The insoluble material was centrifuged off and the supernatant mixed with 3 vol. of absolute ethanol. The waxy precipitate so obtained was dissolved in 300 ml. of chloroform–methanol (1:1, v/v) and shaken well with 60 ml. of 1 mM-$CaCl_2$ to remove water-soluble impurities (Folch, Lees & Sloane-Stanley, 1957). The lower layer was then separated and mixed with 600 ml. of absolute ethanol. The precipitate was redissolved in 300 ml. of chloroform and crude phosphatidylinositol precipitated by the addition of 520 ml. of absolute ethanol. The lipid was dissolved in chloroform–methanol (4:1, v/v) for loading on to the column described below.

Silicic acid chromatography. The method of Hanahan, Dittmer & Warashina (1957) was followed, Mallinckrodt silicic acid (100 mesh, 'suitable for chromatography') was used mixed intimately with half its weight of Hyflo Supercel (Johns–Manville, U.S.A.). The lipids eluted with chloroform–methanol (4:1, v/v) were rejected. The fractions containing the first peak eluted by chloroform–methanol (3:2, v/v) were concentrated *in vacuo* to give a product with N:P molecular ratio approximately 0·2:1·0. Most of the pigmented material was removed in the early fractions obtained with chloroform–ethanol (4:1, v/v), so that the final product was almost white.

Hydrolysis of phosphatidylinositol and preparation of glycerylphosphorylinositol. The product from the silicic acid column was hydrolysed for 30 min. at 37° in methanolic 0·5N-NaOH and the diester prepared by chromatography on Dowex 1 as previously described (Hawthorne & Hübscher, 1959*a*). Only the central fractions of the peak corresponding to glycerylphosphorylinositol were worked up so as to avoid any contamination with diesters, such as glycerylphosphorylserine, which could give rise to glycerophosphate on hydrolysis. As will be seen below, in the final distillation and freeze-drying to remove formic acid, care was necessary to avoid partial decomposition of glycerylphosphorylinositol. It was essential to concentrate *in vacuo* at a bath temperature of 50° or less. When the volume had been reduced to 100 ml. the remaining water was removed by freeze-drying in a simple bench apparatus, consisting of a round-bottom 500 ml. flask attached by a ground joint to a trap cooled in solid CO_2 and acetone. This in turn was attached to a soda-lime trap, to absorb traces of formic acid, and finally to a high-vacuum pump.

The syrup obtained after freeze-drying was stored overnight in a vacuum desiccator over solid KOH to remove the last traces of formic acid and then dissolved in about 2 ml. of water/50 mg. of syrup. The solution was neutralized (pH 7·5) with saturated aqueous barium hydroxide and the barium salt precipitated by the addition of 10 vol. of acetone. After storage overnight at 4°, the white precipitate was centrifuged off and taken up in 2 ml. of water. Any insoluble material was spun down and rejected. The clear solution was again treated with acetone and stored overnight at 4°. The pure barium salt of glycerylphosphorylinositol was finally centrifuged down, washed with acetone and ether and stored in a desiccator.

RESULTS

Hydrolysis of different samples of glycerylphosphorylinositol

Since phosphate migration should not take place during the production of glycerylphosphorylinositol from phosphatidylinositol, the proportions of inositol monophosphate and glycerophosphate formed by hydrolysis of the diester can be used in deducing the structure of the original lipid. Hydrolysis with aqueous 0·5N-NaOH at 100° for 40 min. was chosen, because these conditions gave complete breakdown of glycerylphosphorylinositol to monoesters without further decomposition and release of inorganic phosphate.

Hydrolyses of several different preparations of glycerylphosphorylinositol will be described since they illustrate the difficulty of arriving at the true ratio of monoesters liberated.

Hydrolysis of barium salt prepared by Hawthorne & Hübscher (1959*a*). This sample contained 7·2 % of P and had a molar ratio inositol:P:glycerol of 1·0:1:0·82. The barium salt (30 mg.) was hydrolysed with 6 ml. of 0·5N-NaOH for 40 min. at 100°. After cooling and diluting to 30 ml. with water, the hydrolysate was passed through a column of Zeo-Karb 225 (H^+ form, −16+50 mesh; column size 2 cm. × 15 cm.) to remove cations. The column was washed with 200 ml. of water. The combined eluate and washings were made to 250 ml. with sodium tetraborate solution, so that the final borate concentration was 5 mM, and loaded on to a Nalcite SAR column (formate form, 200–400 mesh; 1 cm. × 14 cm.). Elution with 250 ml. of 5 mM-sodium tetraborate–0·10M-ammonium formate removed no phosphate from the column. Fractions (25 ml.) were collected. Two compounds were eluted with 5 mM-sodium borate–0·15M-ammonium formate, in positions corresponding to inositol monophosphate and glycerophosphate (Fig. 1). Of the P eluted 39 % appeared as inositol phosphate. No inorganic P was detected in either peak. The second (glycerophosphate) peak took up 0·39 mole of periodate/g. atom of P, which suggested that it contained 61 % of β-glycerophosphate. The α- and β-glycerophosphate do not separate on the column used. Since only one inositol-containing peak was observed it seems that the isomers of inositol phosphate also run together.

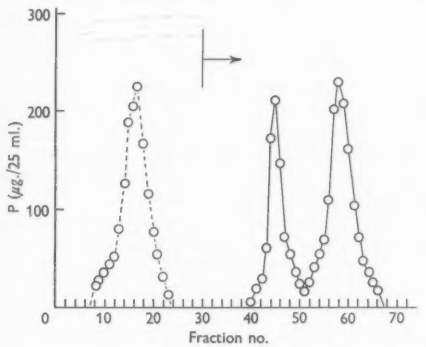


Fig. 1. Column behaviour of glycerylphosphorylinositol and its hydrolysis products. Nalcite SAR column, 25 ml. fractions. Before hydrolysis (broken lines): fractions 8–23, glycerylphosphorylinositol. Hydrolysis products: fractions 40–51, inositol monophosphate; fractions 51–66, glycerophosphate. Eluting mixtures: fractions 1–30, 5 mM-sodium tetraborate–0·06M-ammonium formate; fractions 31–70, 5 mM-sodium tetraborate–0·15M-ammonium formate.

Hydrolysis of the barium salt of glycerylphosphorylinositol and paper chromatography. In these estimations 0·3 ml. of a solution of the barium salt, containing about 250 μg. of P/ml., was heated at 100° for 40 min. with 0·3 ml. of N-NaOH. After cooling, cations were removed with Zeo-Karb 225 and the hydrolysate was concentrated to a small volume for paper chromatography. An amount containing 30–40 μg. of P was applied to each spot on a Whatman no. 54 paper. Markers of inositol 2-phosphate (from phytic acid) and β-glycerophosphate were also applied. Two papers were used for each hydrolysate, one chromatographic solvent being propan-1-ol–aq. NH_3 soln. (sp.gr. 0·88)–water (5:4:1, by vol.) and the other propan-2-ol–acetic acid–water (3:1:1, by vol.) After spraying by the method of Wade & Morgan (1953) the white phosphate-containing areas were cut out. The paper was shredded and washed with water until the pink background surrounding the spots had been completely removed. The extract was then boiled to a small volume and digested in the usual way for the estimation of P. Blanks on similar areas of the chromatogram where there were no spots revealed no P.

In the alkaline solvent both inositol phosphate and glycerophosphate from the hydrolysates ran as double spots. The phosphate determinations were done on the pair together in each case. The larger glycerophosphate spot moved faster and the larger inositol monophosphate spot moved more slowly. Comparison with synthetic α-glycerophosphate (kindly made by Mr B. Clark) showed that the slower glycerophosphate spot corresponded to the α-compound. From the results of Pizer & Ballou (1959) it seems probable that the faster inositol monophosphate spot is the 2-phosphate, the slower being the 1-phosphate. These double spots do not separate if the chromatograms are overloaded.

With the same sample of glycerylphosphorylinositol, five different chromatograms gave 41·1, 41·7, 37·2, 38·4 and 40·9 % of total P of hydrolysate present as inositol monophosphate.

Hydrolysis of glycerylphosphorylinositol without isolation as barium salt. Experiments on partly hydrolysed samples indicated that slight hydrolysis of glycerylphosphorylinositol during its isolation might lead to inaccurate figures for the composition of the final NaOH hydrolysate. Such samples contain inositol monophosphate and glycerophosphate. After acetone precipitation of the barium salts, the former is preferentially precipitated with the glycerylphosphorylinositol.

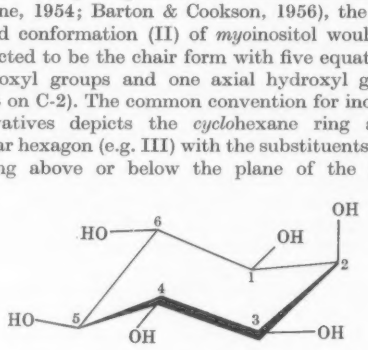
To avoid any decomposition, glycerylphosphorylinositol was separated from a phosphatidylinositol hydrolysate by chromatography on Dowex 1 as described under Materials and Methods. No attempt was made to isolate glycerylphosphorylinositol as a solid. Instead the central peak fractions were pooled, made 0·5N with respect to NaOH and boiled for 40 min. After cooling, cations were removed with Zeo-Karb 225. The resin was washed to avoid losses. The pH of the combined hydrolysate and washings was adjusted to 7 with sodium bicarbonate. Sodium borate solution was then added to give a final borate concentration of 5 mM and a volume of 500 ml. The solution, containing 6·25 mg. of P, was loaded on to the usual Nalcite SAR column. No phosphate was eluted with 0·06M-ammonium formate–5 mM-sodium borate (500 ml.). Two peaks, corresponding to inositol monophosphate and glycerophosphate, were eluted with 0·15M-ammonium formate–5 mM-sodium borate. Of the total P 34 % appeared as inositol monophosphate.

Hydrolysis of synthetic glycerylphosphorylinositol. A sample of the barium salt of glycerylphosphorylinositol was kindly supplied by Mr J. H. Davies and Dr T. Malkin (Davies & Malkin, 1959). It contained 7·3 % of P and the glycerol:P ratio was 1·03:1. Chromatography on Dowex 2 (×10, 200–400 mesh, formate form), by using 0·06M-ammonium formate–5 mM-sodium borate for elution, gave a sharp peak in the first few fractions. This tailed considerably, however, possibly because of hydrolysis on the column. Only the fractions corresponding to the peak were used in further studies. These fractions were combined and hydrolysed directly, as described above. The chromatographic separation was done on a Dowex 2 (×10) column. Of the total P 31 % appeared as inositol monophosphate. A second experiment gave 35 %.

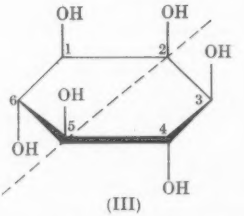
A synthetic sample made by a different method (Ellis & Hawthorne, 1959) was hydrolysed in the same way. It gave 31 % of total P as inositol monophate.

DISCUSSION

From the principles of conformational analysis (Klyne, 1954; Barton & Cookson, 1956), the preferred conformation (II) of *myo*inositol would be expected to be the chair form with five equatorial hydroxyl groups and one axial hydroxyl group (that on C-2). The common convention for inositol derivatives depicts the *cyclo*hexane ring as a planar hexagon (e.g. III) with the substituents projecting above or below the plane of the ring.



(II)



(III)

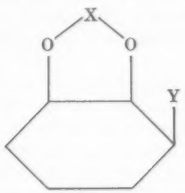
Although these formulae clearly indicate the steric relationships of substituents and are convenient for the application of rules of symmetry, they do not reveal the true shape of the molecule, and care must be exercised in the interpretation of reactions on the basis of such formulae. In this discussion, however, the convention in which the ring is considered planar will be used and the hydroxyl groups described as being in *cis* or *trans* relationship; certain points then become clearer. Use of the non-planar convention does not affect the argument (e.g. Angyal, 1957).

Since the *myo*inositol molecule is symmetrical about the plane perpendicular to the broken line in (III), there are two possible optically inactive monophosphoric acids, the 2- and 5-compounds. The other monophosphoric acids will be pairs of stereoisomers, the 1(3)- and 4(6)-compounds. If it is assumed that the inositol monophosphate isolated from phosphoinositides is optically inactive (see below), it should be the 2- or 5-compound (Iselin, 1949; Hawthorne & Chargaff, 1954). The latter workers considered that cyclization and possible migration of the phosphate group was unlikely to occur in the hydrolysis of phosphoinositides. Recent evidence is that such cyclization does occur (Posternak, 1958; Pizer & Ballou, 1959).

The argument from optical inactivity is not affected, however, by migration, since, if the 2- or 5-phosphate was originally present, migration would lead to mixtures of 2- and 1(3)-phosphate or 5- and 4(6)-phosphate respectively. [As will be seen from (III), the 1- and 3-compounds are optical isomers, so that 1(3)- is equivalent to DL-1-. The same applies to the 4- and 6-phosphate.] These would all be inactive since the 1(3)- and 4(6)-products will be racemic mixtures. On the other hand, if the lipids contain 1- or 4-phosphate, hydrolysis would lead to mixtures still containing active compounds.

The work of Brown *et al.* (1958) on the hydrolysis of glycerylphosphorylinositol analogues [glycerol 1-(2)-hydroxy*cyclo*hexyl phosphate], in which inositol was replaced by *cis*- or *trans-cyclo*hexanediol, showed that mixtures of glycerophosphate and *cyclo*hexanediol phosphate were produced. The *trans* compound gave only $23 \pm 5\%$ of *cyclo*hexanediol phosphate on alkaline hydrolysis, and the *cis* compound gave $86 \pm 2\%$. Brown *et al.* point out that their diesters contained about 10% of the glycerol 2-isomer, which would not be strictly comparable with glycerylphosphorylinositol. More recent work (Posternak, 1958; Pizer & Ballou, 1959) supports the hypothesis of Brown *et al.* that hydrolysis of glycerylphosphorylinositol may take place by either of the routes illustrated in Fig. 2, each involving the intermediate formation of a cyclic triester. On this assumption, glycerylphosphorylinositol having the inositol 5-phosphate structure would behave like the *trans-cyclo*hexanediol compounds and give roughly 23% of inositol monophosphate on hydrolysis.

The situation is more complex in the inositol 2-phosphate structure. By analogy with the *cis-cyclo*hexanediol diester hydrolysis, a high proportion of inositol monophosphate would be expected when glycerol 1-(inositol 2-phosphate) is hydrolysed, since the phosphate residue is flanked by *cis*-hydroxyl groups. However, results from other fields suggest that a further steric effect might be operative. Thus in certain *cyclo*hexane derivatives, reactions which yield a *cis*-fused dicyclic system (IV), or which proceed via an intermediate possessing this five- and six-membered ring system, will be hindered by a group (e.g. OH, $O{\cdot}CO{\cdot}CH_3$, $O{\cdot}CH_3$) which is vicinal and *cis* to the five-membered ring (i.e. Y in IV). The following facts illustrate the



(IV)

operation of this steric effect. *myo*Inositol forms a 1:2-*O*-*iso*propylidene derivative with difficulty (Angyal & Macdonald, 1952), as does *cyclo*hexane-1:2:3-triol (all *cis*) (Christian, Gogek & Purves, 1951), whereas related compounds which contain two but not three vicinal *cis*-hydroxyl groups readily form *iso*propylidene derivatives. Borate ion forms complexes less readily with *cyclo*hexane-1:2:3-triol (all *cis*) than with *cyclo*hexane-1:2/3-triol (only 1:2-*cis*) (Angyal & McHugh, 1957).

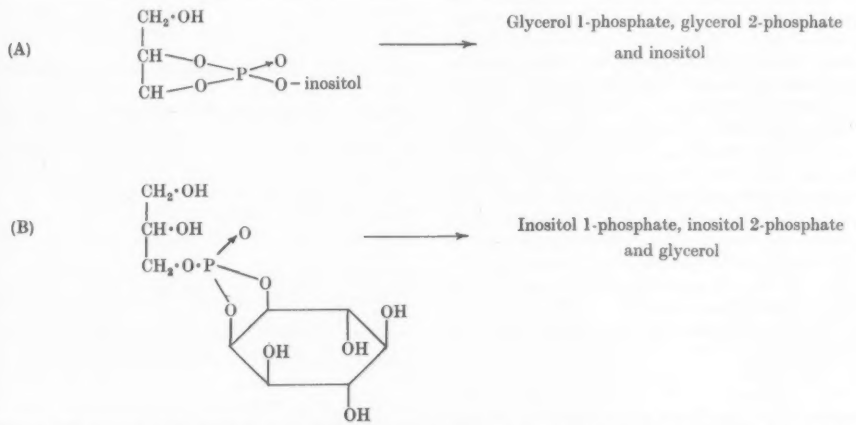


Fig. 2. Mechanism of hydrolysis of glycerylphosphorylinositol. Two possible cyclic intermediates, A and B, with their corresponding hydrolysis products, are shown.

Borate-complex formation with pyranose sugar derivatives which contain vicinal *cis*-hydroxyl groups flanked by a *cis*-methoxyl group is hindered (Foster, 1957). Certain neighbouring group reactions of some sugar acetates are hindered (Lemieux & Brice, 1956), since they involve intermediates structurally related to (IV). A detailed interpretation of these steric effects is given elsewhere (Foster, 1957; Lemieux & Brice, 1956).

Such a steric effect operating in the hydrolysis of glycerol 1-(inositol 2-phosphate) would oppose the formation of an inositol cyclic phosphate and hence divert the hydrolysis along the alternative pathway involving a glycerol cyclic phosphate. Significantly less inositol monophosphate would result from glycerol 1-(inositol 2-phosphate) than *cyclo*hexane *cis*-1:2-diol phosphate from the *cyclo*hexane *cis*-diol analogue.

The observed figures of 30–40 % of inositol monophosphate are therefore quite probably in keeping with the *cis* structure for the inositol phosphate of phosphatidylinositol. Confirmation of this comes from the hydrolysis figures for synthetic glycerol 1-(inositol 2-phosphate.) These agree well with the figures from the natural glycerylphosphorylinositol. Hydrolysing phosphatidylinositol itself, which should give a similar ratio since the fatty acids are quickly removed by alkali, Hanahan & Olley (1958) found 35 % of inositol monophosphate. Lower figures would be expected for a *trans* structure. In this case glycerylphosphorylscyllitol should provide an ideal model compound.

The chemical evidence suggests that the inositol phosphate of liver phosphatidylinositol has its phosphate group on one of the *cis*-hydroxyl groups. It must therefore be the 1-(D- or L-) or 2-phosphate. These structures would give similar hydrolysis results and so cannot be distinguished without further evidence. Until recently the inositol monophosphate isolated from lipids was thought to be optically inactive (Iselin, 1949; Hawthorne, 1955*b*). Pizer & Ballou (1959) have now isolated an inositol monophosphate with a small but definite rotation from soya-bean inositide. From their chemical studies and from this rotation they conclude that soya-bean inositide has the 1-structure. Since this vegetable lipid has strongly dextrorotatory sugar phosphates associated with it (Hawthorne, 1955*b*) it was thought advisable to obtain further evidence on the rotation (and if possible other independent evidence), before deciding between the 1- and 2-structures (see Hawthorne, Kemp & Ellis, 1960).

SUMMARY

1. Liver phosphatidylinositol has been hydrolysed to give glycerylphosphorylinositol, alkaline hydrolysis of which gives a mixture of inositol monophosphate (30–40 %) and glycerophosphate.

2. Hydrolysis of synthetic glycerol 1-(inositol 2-phosphate) gives a similar mixture.

3. Steric effects in the hydrolysis are discussed and it is concluded that liver phosphatidylinositol has the inositol 1- or 2-phosphate structure.

I should like to thank Professor A. C. Frazer for his interest in the work, Dr A. B. Foster for valuable discussion of the conformational aspects of it and Miss M. Bason for expert technical assistance.

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Phosphoinositides

2. THE INOSITOL 1-PHOSPHATE STRUCTURE IN LIVER PHOSPHATIDYLINOSITOL

By J. N. HAWTHORNE, P. KEMP and R. B. ELLIS
Department of Medical Biochemistry and Pharmacology, Medical School, Birmingham 15

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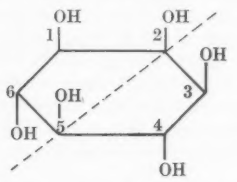
The hydrolysis studies reported in the preceding paper (Hawthorne, 1960) suggest that in liver phosphatidylinositol the phosphate group is linked to one of the *cis*-hydroxyl groups of the inositol, i.e. to positions 1(3)- or 2-. Until recently, the inositol monophosphate isolated from phosphoinositides was believed to be optically inactive (Iselin, 1949; Hawthorne, 1955) and so liver phosphatidylinositol was thought to have the 2-structure (Hawthorne & Hübscher, 1959*a*). It can be seen from the structure (I) that only the 2- or 5-phosphate has



(I)

a plane of symmetry. Pizer & Ballou (1959) however, have shown the inositol monophosphate from soya-bean phosphoinositide to be optically active. The same conclusion has been reached by Hanahan & Brockerhoff (1959) for liver phosphatidylinositol. The subject has therefore been reinvestigated with optical rotatory dispersion to study the optical activity of carefully purified inositol monophosphate from groundnut phosphatides, and an enzymic hydrolysis of liver phosphatidylinositol which should release inositol monophosphate without phosphate migration.

MATERIALS AND METHODS

Phospholipids. Liver phosphatidylinositol was prepared as described by Hawthorne (1960). Crude groundnut phosphoinositide, which is similar to the soya-bean product, was also made by the method of Hawthorne (1955).

Enzyme preparation. The phospholipase which hydrolyses phosphatidylinositol was prepared from rat liver as described by Kemp, Hübscher & Hawthorne (1959).

Experiments with Acetobacter. Attempts to oxidize various inositol phosphates were made with a strain of *Acetobacter suboxydans* (A.T.C.C. 621) in the Warburg apparatus (Magasanik & Chargaff, 1948). The most vigorous oxidation of inositol standards was obtained with bacteria grown for 3 or 4 days (not longer) on the sorbitol–yeast extract medium. For the oxidations, acetate buffer of pH 5·6 was used. Inositol phosphates in the form of *cyclo*hexylamine salts were first treated with Zeo-Karb 225 and the free acid so produced was neutralized with NaOH.

Other analytical methods and ion-exchange resins. These have been described by Hawthorne & Hübscher (1959*b*).

RESULTS

Preparation of inositol monophosphate from groundnut phosphoinositide. The material was hydrolysed with KOH according to Pizer & Ballou (1959). The *cyclo*hexylamine salt finally obtained was not recrystallized but applied directly to a column of De-Acidite FF (The Permutit Co. Ltd., London). The salt had 4·95 % of P and $[\alpha]_D^{20} + 26 \pm 2°$ in water (*c*, 2). It was Molisch-positive. A portion (1·15 g.) was applied to a column of resin (200–400 mesh; size 3 cm. × 30 cm.). Elution with 0·01 M-formic acid gave three peaks. The first two were due to sugar-containing compounds, but the third compound had only a trace of sugar. Rotations of this last compound, calculated as the *cyclo*hexylamine salt, were $[\alpha]_D^{20} - 2·7 \pm 0·2°$ (pH 1) and $[\alpha]_D^{20} + 5·6 \pm 0·2°$ after addition of *cyclo*hexylamine to pH 9 (*c*, 1·53).

For further purification, the fractions corresponding to the third peak were combined and sodium borate solution was added to give a final concentration of 5 mM. The combined fractions, containing 11 mg. of P, were then applied to a Dowex 2 (× 10) column (formate form, 100–200 mesh; 1·5 cm. × 35 cm.). All the phosphate was retained by the column and none was eluted with 500 ml. of 0·08 M-ammonium formate–5 mM-sodium borate, nor with 1 l. of 0·12 M-ammonium formate–5 mM-sodium borate. Organic P (8·5 mg.) was eluted as one peak with 0·15 M-ammonium formate–5 mM-sodium borate. This solvent removes inositol monophosphate. The central fractions of the peak were pooled, diluted with an equal volume of 5 mM-borate and applied to a similar Dowex 2 column. One main peak was then eluted with 0·05 M-formic acid–0·05 M-ammonium formate. Central fractions were pooled and cations removed with Zeo-Karb 225. The solution thus obtained was concentrated to 50 ml. *in vacuo* at 30° and then

freeze-dried. The residue was taken up in 2 ml. of water and neutralized with saturated barium hydroxide solution. The barium salt of inositol monophosphate was precipitated with excess of acetone. The white solid was centrifuged and re-precipitated from water with acetone. To its solution in a little water the calculated amount of *cyclo*hexylamine sulphate solution was then added. Barium sulphate was spun down and the clear supernatant shaken with excess of butan-1-ol. Addition of 1 vol. of diethyl ether gave a semi-crystalline precipitate of *cyclo*hexylamine inositol monophosphate. This was recrystallized in the same way to give small crystals of the salt.

Inositol monophosphate from phytic acid. For comparison, the crystalline *cyclo*hexylamine salt of inositol monophosphate prepared enzymically from phytic acid was made (McCormick & Carter, 1952). This is believed to be the 2-phosphate (Hawthorne, 1955; Brown & Hall, 1959).

Rotation measurements. For the groundnut inositol monophosphate as the *cyclo*hexylamine salt, the value $[\alpha]_D^{20} + 3{\cdot}1 \pm 0{\cdot}3°$ was obtained. The phytic acid compound showed no optical activity. Samples of each salt were sent to Dr W. Klyne for rotational-dispersion measurements. The groundnut compound gave these molecular rotations (wavelengths are given in parentheses): $+12°$ (600 mμ); $+18°$ (500 mμ); $-32°$ (400 mμ); $-54°$ (350 mμ); $-89°$ (300 mμ). At each of these wavelengths the phytic acid compound gave values of zero within the error of the instrument. In all the above-mentioned measurements water was used as solvent and calculations were based on the di*cyclo*hexylamine salts.

Studies with Acetobacter suboxydans. Attempts were made to oxidize synthetic (−)-inositol 3-phosphate (Kilgour & Ballou, 1958), *myo*inositol 2-phosphate (from phytic acid) and the groundnut inositol phosphate. In each experiment inositol standards were oxidized to make sure that the bacteria were active. No oxidation was observed with any of the inositol phosphates.

Enzymic hydrolysis of phosphatidylinositol. A sample of phosphatidylinositol equivalent to about 40 mg. of P was emulsified in water and dialysed to remove non-lipid phosphates. To this was added the enzyme extracted from five rat livers as described previously (Kemp *et al.* 1959); the precipitate obtained at 40% saturation of $(NH_4)_2SO_4$ was dissolved in 100 ml. of 0·01 M-2-amino-2-hydroxymethylpropane-1:3-diol–hydrochloric acid buffer, pH 7·3. 0·4 M-Sodium acetate buffer, pH 5·4 (20 ml.), and 20 ml. of 0·08 M-$CaCl_2$ were also added. The final volume was 220 ml. The mixture was incubated for 75 min. at 37° and then 25 ml. of 50% (w/v) trichloroacetic acid solution and some crushed ice were added. After 10 min. at 4°, 50 ml. of an aqueous 12·5% (w/v) suspension of Norit charcoal was added and the mixture shaken for 10 min. It was then centrifuged at 2000 rev./min. The precipitate was washed in the centrifuge with four 50 ml. portions of water. These were then added to the original supernatant.

The supernatant and washings were extracted four times with 100 ml. of diethyl ether to remove trichloroacetic acid. Excess of ether was removed by a stream of air, and cations were removed by shaking with Zeo-Karb 226 (H^+ form). Sodium borate solution and deionized water were then added to give a borate concentration of 5 mM in a final volume of 2 l.

Isolation and paper chromatography of inositol monophosphate. The above-mentioned solution was loaded on to a Nalcite SAR column which was then washed with 500 ml. of 5 mM-sodium borate. No phosphate was eluted with this or with 500 ml. of 0·06 M-ammonium formate–5 mM-sodium borate. Of the phosphate applied 90% was eluted as one peak as soon as the solvent was changed to 0·15 M-ammonium formate–5 mM-sodium borate. This peak corresponded in position with inositol monophosphate. The peak fractions were combined and re-applied to the same type of column for borate and formate removal (Hawthorne & Hübscher, 1959*b*). During elution from this second column a small peak corresponding to about one-tenth of the total P was seen in front of the main peak. It was rejected.

After removal of the last traces of formic acid in a vacuum desiccator over KOH, the inositol monophosphate was dissolved in a little water. Portions corresponding to 10 μg. of P were applied to Whatman no. 54 papers for descending chromatography in propan-1-ol–aq. NH_3 soln. (sp.gr. 0·88)–water (6:3:1, by vol.). The solvent was allowed to run off the end of the papers for several hours. Markers of inositol 2-phosphate and the mixture of inositol 1- and 2-phosphate produced by acid-catalysed migration (Posternak, 1959) were applied. Only a trace of 2-phosphate was seen in the enzymically produced inositol monophosphate. The bulk of the compound ran to the inositol 1-phosphate position. The 2-compound may have been produced during the formic acid removal.

DISCUSSION

The results suggest that the inositol monophosphate from groundnut phosphoinositides is optically active. The values obtained ($[\alpha]_D + 3{\cdot}1°$ for the *cyclo*hexylamine salt) are similar to those of Pizer & Ballou (1959) for the soya-bean compound ($+3{\cdot}4°$). In view of the other evidence supplied by these authors it seems likely that both these phosphoinositides have the inositol 1-phosphate structure.

Since a vigorous alkaline hydrolysis is used in the preparation of the monophosphates, some phosphate migration to the 2-position occurs. However, as Pizer & Ballou show, the inositol 2-phosphate can be removed by repeated crystallization because its *cyclo*hexylamine salt is more soluble than that of the 1-phosphate. Under acid conditions (Posternak, 1959), the 2-phosphate itself undergoes phosphate migration, giving a mixture of 1(3)- and 2-phosphate. This further migration would reduce the optical activity of the soya-bean and groundnut products by producing 1(3)-phosphate, but fortunately it does not occur readily under alkaline conditions.

Strongly dextrorotatory sugar phosphates are also present in hydrolysates of these lipids (Klenk & Sakai, 1939; Hawthorne & Chargaff, 1954). The presence of traces of such phosphates could easily mask the small rotation of inositol monophosphate, since the free acid is laevorotatory, and this is presumably why in earlier work (Iselin, 1949; Hawthorne, 1955) the inositol monophosphate was thought to be optically inactive.

Although phosphatidylinositol may occur in soya-bean phosphoinositides (Hörhammer, Wagner & Hölzl, 1958), more complex inositol-containing lipids are also present (Folch, 1952). Conclusions about the inositol monophosphate from groundnut or soya-bean phosphatides therefore cannot be applied directly to liver phosphatidylinositol.

Acetobacter suboxydans of the strain used in these experiments can oxidize (−)-inositol to a diketone, attacking the two axial hydroxyl groups. The bacteria are unable to oxidize (−)-inositol 3-phosphate, even though in this compound the axial hydroxyl groups remain free. This confirms the earlier view (Hawthorne, 1955) that the phosphate group prevents enzymic oxidation whether it is linked to the hydroxyl group that is usually oxidized or not. Since neither *myo*inositol 1- nor 2-phosphate will be attacked, the bacteria are of no use in structural studies.

Alkaline hydrolysis of phosphatidylinositol or glycerylphosphorylinositol gives a mixture of inositol monophosphate and glycerophosphate in proportions suggesting that the inositol of the original lipid is phosphorylated in the 1- or 2-position (Hawthorne, 1960). Paper chromatography of such hydrolysates shows the presence of inositol 1- and 2-phosphate. By analogy with the behaviour of glycerophosphate the most probable explanation is the intermediate formation of a cyclic inositol 1:2-phosphate during hydrolysis and then 'non-specific' ring opening. Thus it is impossible to decide on the original position of the phosphate group from the results of alkaline hydrolysis.

Similar conditions for cyclic ester formation are present during the action of ribonuclease on ribonucleic acid and a cyclic phosphate mechanism has been proposed for the enzymic hydrolysis. The final products, however, include only nucleoside 3-phosphates and not a mixture of the 2- and 3-compound. It was hoped that a similar specificity might be found in the action of the phospholipase attacking phosphatidylinositol, which is also a phosphodiesterase. This proved to be so. The isolation of inositol 1-phosphate from the enzymic digest therefore provides further evidence that liver phosphatidylinositol has the 1-structure. The work of Hanahan & Brockerhoff (1959), on periodate oxidation of glycerylphosphorylinositol from the same lipid, also leads to this conclusion.

SUMMARY

1. Carefully purified inositol monophosphate from an alkaline hydrolysate of groundnut inositides was optically active.

2. This inositol monophosphate, inositol 2-phosphate and (−)-inositol 3-phosphate were all resistant to oxidation by *Acetobacter suboxydans* (A.T.C.C. 621).

3. The enzymic hydrolysis of liver phosphatidylinositol released inositol 1-phosphate.

4. It is concluded that liver phosphatidylinositol has the inositol 1-phosphate structure.

We wish to thank Professor A. C. Frazer for his continued interest. Dr W. Klyne, of the Postgraduate Medical School, London, kindly undertook the rotatory-dispersion studies. Mrs Janet Day made the measurements. We are indebted to Dr C. E. Ballou and Dr G. L. Kilgour, Biochemistry Department, University of California, for the generous sample of (−)-inositol 3-phosphate. Miss M. Bason provided valuable technical assistance and Mr M. G. Rumsby made the *Acetobacter* studies.

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Mitochondrial Preparations from the Leaves of Tobacco (*Nicotiana tabacum*)

2. OXIDATIVE PHOSPHORYLATION*

By W. S. PIERPOINT
Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts

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A sucrose solution is suitable for the isolation of functional mitochondria from some animal tissues (Hogeboom, Schneider & Pallade, 1948), but not sufficient for use in the isolation of mitochondria from leaves. Smillie (1955), working with pea leaves, added ethylenediaminetetra-acetic acid; James & Das (1957), working with bean leaves, added phosphate and magnesium as well; Pierpoint (1959), working with tobacco leaves, increased the buffering power and added various substances, of which citrate was the most important. Even with these additions the tobacco mitochondria appeared to be somewhat functionally damaged and were heavily contaminated with chloroplast fragments.

Attempts are being made both to assess the damage done to these tobacco-leaf mitochondria and to purify them further. This paper provides information about their respiratory quotients and demonstrates that the oxidations catalysed by them are 'loosely' coupled to the phosphorylation of adenosine nucleotides.

EXPERIMENTAL

Mitochondrial preparations. Two modifications were made to the procedure already described (Pierpoint, 1959). The pH of the extraction medium (sucrose, 0·4M; 2-amino-2-hydroxymethylpropane-1:3-diol (tris), 0·2M; potassium phosphate, 0·01M; ethylenediaminetetra-acetic acid, 5 mM; sodium citrate, 0·02M) was decreased to 7·7, and the amount of medium used was increased so that 25–30 g. of leaves was disrupted in 100–130 ml.

Oxygen uptake. Oxygen uptake was measured in the conventional Warburg apparatus. Details of the procedure, including the incubation temperature (30°), were the same as those described by Pierpoint (1959).

In experiments in which only gas exchanges were being followed the samples of the mitochondrial preparations were added to manometer flasks containing: substrate acid, 40 μmoles; sucrose, 0·3 m-mole; phosphate buffer, pH 7·3, 0·1 m-mole; magnesium sulphate, 10 μmoles; adenosine triphosphate (ATP), 2 μmoles; cytochrome *c*, 0·018 μmole; diphosphopyridine dinucleotide (DPN), 0·2 μmole; thiamine pyrophosphate, 0·2 μmole; coenzyme A (CoA), 0·025 μmole. The final volume was 1·5 ml. and the pH was 7·2–7·3.

When both phosphorylation and oxygen uptake were being followed, the medium in the manometer flasks contained: substrate acid, 40 μmoles; sucrose, 0·4 m-mole; phosphate buffer, pH 7·3, 12 μmoles; magnesium sulphate, 10 μmoles; manganese sulphate, 0·1 μmole; sodium fluoride, 15 μmoles; ATP, 2 μmoles; cytochrome *c*, 0·018 μmole; DPN, 0·2 μmole; thiamine pyrophosphate, 0·2 μmole; glucose, 10 μmoles; hexokinase (Sigma Chemical Co., type II), 1 mg. CoA (0·025 μmole) was also present in most experiments. The final volume varied a little, but was generally 2·0–2·1 ml.

Evolution of carbon dioxide. Evolution of the gas was measured manometrically by the 'direct method' (Umbreit, Burris & Stauffer, 1957).

Orthophosphate disappearance. Samples (1 ml.) were taken from the contents of manometer flasks and added to centrifuge tubes containing 1 ml. of cold trichloroacetic acid (5%, w/v). The tubes were cooled in ice–water for 10 min. and then centrifuged at 1500 *g* for the same length of time. The supernatant solution was decanted, diluted with four times its volume of sodium acetate (0·1M) and portions were taken for phosphate estimation.

Phosphate esterification occurred in the manometer flasks during the equilibration period, before it was possible to follow oxygen uptake. In measuring P/O ratios (μmoles of P esterified/μg.atom of oxygen uptake) it was necessary

* Part I: Pierpoint (1959).

to correct for this by removing some manometer flasks for estimation of P immediately after the equilibration period. Measurement of the adenosine triphosphatase activity occurring in the equilibration period was obtained in the same way.

In calculating P/O ratios, allowance was made for endogenous oxygen uptake and adenosine triphosphatase activity, both of which were assumed to continue unaffected in the presence of substrate. The correction for the enzyme activity was in most cases very small; that for endogenous oxygen uptake was larger and raised the P/O ratio for succinate oxidation by 10%. The main reason for assuming that endogenous uptake was not affected by substrate-stimulated respiration is that it was brought about by a different mechanism. It was, for example, unaffected by cyanide and other inhibitors of succinoxidase, and was unaccompanied by phosphate disappearance.

Chemical estimations. When labile organic phosphates were present, orthophosphate was measured by the method of Lowry & Lopez (1947). Otherwise, as in fractions from chromatographic experiments, the more sensitive methods of Holden & Pirie (1955) were used for orthophosphate and total phosphate.

Protein N was estimated by a micro-Kjeldahl method (Pierpoint, 1959).

Chromatography of phosphates. The contents of the manometer flasks were deproteinized with an equal volume of 5% (w/v) trichloroacetic acid, and the protein was removed by centrifuging (1500 *g* for 10 min.). The supernatant was extracted twice with ether, adjusted to pH 7 with ammonia solution, and, after dilution to 15 ml. with mM-ammonia solution, was added to a column (7 cm. × 1·2 cm.) of Dowex 2 (100–200 mesh; × 8 cross-linkage). The column was further washed with mM-ammonia solution and the phosphates were eluted with solutions similar to those used by Khym & Cohn (1953). Eluent 1 (ammonium chloride, 0·025M; potassium borate, 0·01M; pH 8·4) eluted orthophosphate; eluent 2 (ammonium chloride, 0·03M) eluted glucose 6-phosphate; eluent 3 (potassium chloride, 0·2M; hydrochloric acid, 0·02M) eluted adenosine nucleotides in a single peak.

Radioactive measurements. ^{32}P radioactivity in fractions (10 ml.) from the chromatographic columns was measured in an M6 Geiger counter. The counting period was 10 min. and the results were corrected for 'lost counts' and background activity. As the estimations in each experiment were done on the same day, no correction for radioactive decay was made.

Chemicals. Phosphate buffers were prepared from M-NaH_2PO_4 by adding sodium hydroxide soln. and diluting them to the required molarity. Tris buffers were similarly made from 2-amino-2-hydroxymethylpropane-1:3-diol and hydrochloric acid. The solutions were finally adjusted to the required pH values after dilution.

RESULTS

Respiratory quotients for the oxidation of organic acids

Respiratory quotients for the oxidation of a number of tricarboxylic acid-cycle substrates (Table 1) are similar to those reported for mitochondrial preparation from other plant sources, such as soya-bean hypocotyls (Switzer & Smith, 1957). As with soya-bean preparations the low value for succinate was probably due to the slow rate at which fumarate was oxidized. The similarity of the other R.Q. values to those expected for complete oxidation of substrates of the tricarboxylic acid cycle is probably coincidental, for during the oxidation of these substances, other acids accumulate (Pierpoint, 1959).

With pyruvate and malonate as substrates there was neither uptake of oxygen nor evolution of carbon dioxide.

Oxidative phosphorylation

Disappearance of orthophosphate accompanying the oxidations. In the presence of a number of cofactors, orthophosphate disappeared during the mitochondrial oxidation of succinate (Fig. 1). This phosphate removal started during the 10 min. when the manometer flasks were equilibrating and proceeded almost linearly with time until nearly all the orthophosphate had gone. Orthophosphate did not disappear when the leaf preparations were briefly boiled before incubation, or when substrate was absent. Without substrate the orthophosphate concentration often increased slightly (1–5%), probably because of the action of adenosine triphosphatases.

The phosphorylation reactions that led to the disappearance of phosphate were 'loosely' coupled (Remmert & Lehninger, 1959) to the oxidative reactions. Omission of some substances from the incubation medium considerably diminished the phosphorylation, although this had little effect on oxygen uptake. In particular, ATP or ADP, hexokinase and glucose, substances that might be expected to accept the phosphate groups of unstable esters, were necessary for high phosphorylation rates (Table 2). In the absence of glucose and hexokinase the adenosine triphosphatase activity was increased about eightfold, masking much of the phosphate disappearance.

Table 1. *Respiratory quotients for the oxidation of acids by mitochondrial preparations*

The gas exchanges catalysed by mitochondrial preparations were measured over 2 hr. periods, both in the presence and absence of substrates. Allowances were made for the endogenous activity in calculating respiratory quotients for the oxidation of the substrates.

Substrate	Endogenous respiratory quotient	Respiratory quotient in the presence of substrate
Citrate	0·21	1·5
α-Oxoglutarate	0·26	1·4
Succinate	0·20	0·28
Malate	0·20	1·4

Neither DPN nor triphosphopyridine nucleotide had any effect on oxygen uptake or phosphate removal.

Occasional mitochondrial preparations would catalyse oxidation but not phosphate removal. Invariably such preparations had endogenous oxygen uptakes that were at least a quarter of the uptake in the presence of succinate. This high ratio of endogenous to succinoxidase activity, like the loss of phosphorylating powers, is probably a symptom of mitochondrial damage, and may be a useful indicator of it.

P/O ratios for the oxidation of organic acids. Table 3 shows the P/O ratios obtained for the oxidation of tricarboxylic acid substrates by the tobacco-leaf preparations. No attempt was made to limit these oxidations to single steps of the tricarboxylic acid cycle, so that the P/O ratio values

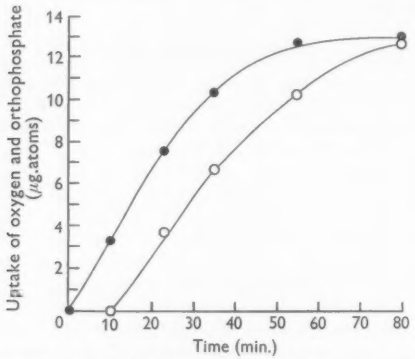

Fig. 1. Orthophosphate disappearance and oxygen uptake from mitochondrial preparations. Samples of the mitochondrial preparations (0·6 mg. of protein N) were incubated in manometer flasks containing the medium described in the Experimental section (final vol. 1·7 ml.; CoA omitted). Oxygen uptake (○) was followed after a 10 min. equilibration period, and samples were removed at various times for phosphate estimation (●). A series of control manometers, without succinate, were used to measure endogenous oxygen uptake and appearance of phosphate due to adenosine triphosphatases. The values plotted have been corrected for both these changes.

Table 2. *Effect of components of the incubation medium on phosphorylation*

Samples of the mitochondrial preparations (0·6–0·95 mg. of protein N) were incubated with succinate as described in the Experimental section. The medium contained triphosphopyridine dinucleotide (TPN, 0·1 μmole), half the stated quantities of cytochrome *c*, thiamine pyrophosphate, DPN and orthophosphate, and it was modified as described below. Oxygen uptake was followed for 30 min., when the reactions were stopped and samples taken for orthophosphate estimations. The values given below have been corrected for endogenous oxygen uptake, and for adenosine triphosphatase activity. AMP, Adenosine monophosphate.

Expt. no.	Modification to medium	Oxygen uptake (%)	Phosphorylation (%)
1	None	100	100
	ATP omitted	95	25
	ADP replaced ATP	110	100
2	ADP replaced ATP	100	100
	AMP replaced ATP	110	50
	ATP omitted	106	30
3	None	100	100
	Glucose omitted	100	100
	Hexokinase omitted	120	54
	Glucose and hexokinase omitted	115	28
4	None	100	100
	TPN, fluoride and manganese sulphate omitted	97	90

Table 3. P/O *ratios for the oxidation of organic acids*

The ability of samples (0·5–1·0 mg. of protein N) of mitochondrial preparations to take up oxygen and orthophosphate was measured over a 30 min. period as described in the Experimental section. Hexokinase was, however, omitted from the medium in Expt. 5, and the substrate concentrations were altered in Expt. 6. The values given are corrected for endogenous oxygen uptake and adenosine triphosphatase activity.

Expt. no.	Substrate	Oxygen uptake (μg.atoms)	Orthophosphate disappearance (μmoles)	P/O ratio
1	Succinate	6·1	8·0	1·3
2	Malate	6·2	7·8	1·3
	Citrate	2·6	3·8	1·5
3	Fumarate	3·8	5·0	1·3
4	Isocitrate	3·0	6·3	2·1
	Isocitrate plus TPN (0·2 μmole)	3·4	6·2	1·8
5	α-Oxoglutarate	6·9	6·5	0·9
6	Malate (14 μmoles)	4·5	9·6	2·1
	DPNH (9 μmoles)	4·0	1·3	0·3

may be the result of a number of oxidative and phosphorylative reactions.

The P/O ratio values for the oxidation of α-oxoglutarate were measured in the absence of hexokinase. They are not therefore strictly comparable with the other values in Table 3, or with higher values obtained for pea-leaf preparations (Smillie, 1956). Hexokinase was omitted as it inhibited oxygen uptake from α-oxoglutarate by 90%. Glucose and fluoride were also inhibitory but to a smaller (30%) degree. The mechanism of these inhibitions is obscure, but that due to hexokinase and glucose may reflect a requirement of ATP for the oxidation. ATP stimulates the oxidation of α-oxoglutarate although it has no effect on succinate oxidation (Pierpoint, 1959).

The P/O ratio given in Table 3 for the oxidation of reduced DPN (DPNH) is much lower than that for the DPN-dependent oxidation of malate obtained in the same experiment. Similar low values have been observed with animal preparations. They have been attributed to permeability barriers preventing the free diffusion of DPNH (Lehninger, 1951), and the inhibition of phosphorylation by the DPN formed (Lehninger, 1949). Higher P/O values (0·6–0·7) were obtained with tobacco-leaf fractions when lower concentrations of DPNH were used.

Both isocitrate and fumarate caused bigger oxygen uptakes (Table 3) than those previously recorded (Pierpoint, 1959). The improvement in the first case was due to the use of isocitrate prepared by Dr H. B. Vickery (Vickery & Wilson, 1958). It was oxidized ten times as rapidly as was a commercial DL sample of this substances. The increased oxidation of fumarate is commented on in the next paper (Pierpoint, 1960).

Bovine serum albumin stabilizes some enzymes of plant mitochondria (Price & Thimann, 1951), and improves oxidative phosphorylation in insect mitochondria (Sacktor, O'Neill & Cochran, 1958). It was therefore tested against the tobacco mitochondria. It diminished both the oxidation of succinate and the accompanying phosphorylations by a third, leaving the P/O ratio unaltered.

Effect of 2:4-*dinitrophenol on phosphate disappearance.* 2:4-Dinitrophenol (DNP) inhibited phosphate removal by tobacco-leaf preparations. This inhibition did not result in increased oxygen uptake, which often occurs when oxidation and phosphorylation are more tightly coupled. In fact oxygen uptake was inhibited also, although to a smaller degree (Fig. 2). Phosphate disappearance associated with α-oxoglutarate oxidation was just as sensitive to DNP as that with other substrates (Table 4).

The adenosine triphosphatases of tobacco-leaf preparations, like those of a similar preparation from mosquito (Avi-dor & Gonda, 1959), were unaffected by DNP. The inhibition of phosphorylation by this compound was not therefore a consequence of the acceleration of the breakdown of phosphate esters.

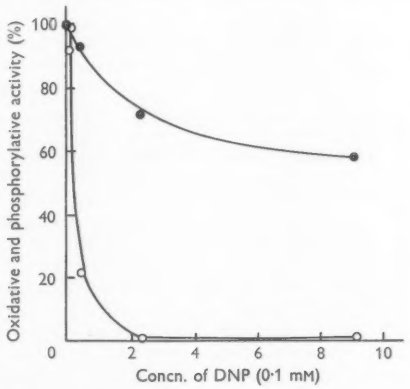

Fig. 2. Effect of DNP on mitochondrial activities. Samples of tobacco-leaf preparations (0·65 mg. of protein N) were incubated as described in the Experimental section in the presence of succinate and various amounts of DNP. Oxygen uptake (●) was followed for 30 min., when the reactions were stopped and orthophosphate disappearance (○) was estimated. Corrections were made for endogenous oxygen uptake, adenosine triphosphatase activity and phosphate disappearance during the equilibration period.

Table 4. *Effect of dinitrophenol on the oxygen and phosphorus metabolism of tobacco-leaf preparations*

Mitochondrial preparations were incubated as described in the Experimental section, either in the presence or the absence of DNP (0·14 mM). Oxygen and phosphorus uptakes were measured over a 30 min. period and corrected for endogenous oxygen uptake and adenosine triphosphatase activity.

Substrate	Inhibition of oxygen uptake (%)	Inhibition of phosphorus disappearance (%)	P:O ratio	
			DNP absent	DNP present
Succinate	20	96	0·7	0·03
Malate	20	90	1·3	0·15
Isocitrate	50	100	2·1	0
α-Oxoglutarate	14	95	0·9	0·05

Accumulation of phosphate esters during oxidation. The reactions leading to the disappearance of orthophosphate are those involved in the formation of adenosine nucleotides and glucose 6-phosphate. The accumulation of both these phosphate esters during the oxidation of succinate is shown in Fig. 3. The mitochondrial preparation used in this experiment esterified about 40% of the added phosphate, with a P/O ratio of 0·95. The amount of phosphate formed due to adenosine triphosphatases was only a small (5%) proportion of the total present. The chromatographic analyses show the formation of a little glucose 6-phosphate at the expense of ATP when the incubation was done in the absence of substrate. When succinate was present, however, there was a larger increase in the amount of glucose 6-phosphate as well as a small increase in nucleotide phosphate. Additional evidence that the disappearing orthophosphate was incorporated into nucleotide phosphate was obtained from experiments in which radioactive orthophosphate was used and the glucose and hexokinase were omitted from the reaction medium. Fig. 4 summarizes the results of one such experiment. The mitochondrial preparation had the high adenosine triphosphatase characteristic of preparations tested in the absence of glucose and hexokinase. During incubation in the absence of substrate, the orthophosphate increased by a third. This breakdown of ATP is shown in the top graph of Fig. 4, as well as the decrease (20%) in the specific activity of orthophosphate that results from it. When substrate was present during the incubation there was no change in

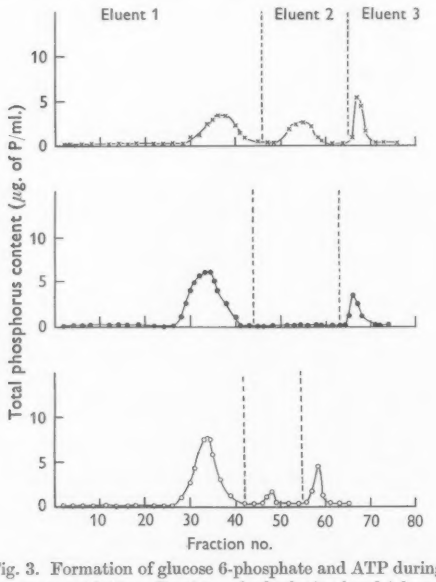

Fig. 3. Formation of glucose 6-phosphate and ATP during succinate oxidation. Portions of a leaf-mitochondrial preparation (0·8 mg. of protein N) were suspended in the medium described in the Experimental section, except that the concentration of phosphate was increased to 18 μmoles and that of ATP decreased to 1·2 μmoles. Incubation at 30° in manometer flasks was carried out for 0 hr. (●) and 1·25 hr. (×) in the presence of substrate, and 1·25 hr. in its absence (○). The contents of duplicate flasks were pooled for chromatography. Total P, orthophosphate and $E_{260\,m\mu}$ were measured on samples from the resulting fractions, but only total P is shown in the graph. The peaks of phosphorus in the top graph represent, from the left, orthophosphate, glucose 6-phosphate and nucleotide phosphates respectively.

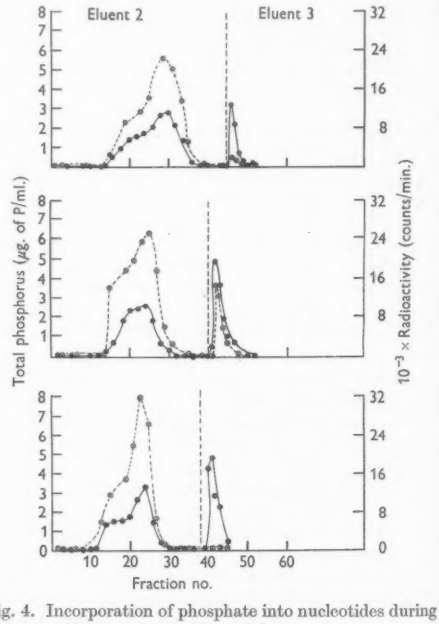

Fig. 4. Incorporation of phosphate into nucleotides during malate oxidation. A mitochondrial preparation was suspended in sucrose (0·25M) and divided into three lots (2 mg. of protein *N*). One (middle graph) was added to the medium described in the Experimental section, plus [^{32}P]-orthophosphate (2 μc) and malate (40 μmoles). The second (bottom graph) was boiled for 5 min. and then added to the same medium. The third (top graph) was added to the medium from which malate had been omitted. All were incubated at 30° for 40 min., deproteinized and then chromatographed. Samples of the fractions collected (10 ml.) were taken for total phosphate estimations (●) and radioactivity measurements (⊗). The two peaks of phosphate represent orthophosphate, on the left, and nucleotide phosphates.

the concentration of nucleotide phosphates, and these substances became radioactive with a specific activity about a third of that of orthophosphate. Fig. 4 also shows that there is a small incorporation of radioactivity into nucleotides in the absence of substrate. Such an orthophosphate–ATP exchange reaction is well known in animal mitochondria and their fragments, and is thought to reflect some transphosphorylation reaction of the oxidative-phosphorylation process (Cooper & Lehninger, 1957). With the tobacco-leaf preparations, however, it might equally well be due to the oxidation of traces of citrate derived from the isolation procedure, or to photosynthetic phosphorylation (Arnon, Whatley & Allen, 1958) associated with the chloroplast fragments.

The asymmetric shape of some of the orthophosphate peaks in Fig. 4 was not caused by contaminating phosphates. The specific activities of fractions taken from different parts of these peaks were identical. Moreover, the same asymmetry was observed when authentic orthophosphate was eluted from these resins with 0·03 M-ammonium chloride. It may result from the presence of different ionic species of orthophosphate, or from interference caused by bicarbonate contamination of the alkaline eluents (Björk & Boman, 1959).

Stability of oxidation and phosphorylation reactions. Once the mitochondrial preparations were centrifuged from the disintegrated leaves, washed and suspended in a sucrose medium, they were stable enough for convenient experimentation. When, for example, they were suspended in the substances usually added to the manometer flasks and incubated at 30° without substrate, there was no measurable loss in ability to oxidize succinate after 30 min. and over 70 % of the phosphorylative power remained. Longer incubations emphasized the difference in stability between the oxidative and the phosphorylative reactions (Table 5) and revealed a beneficial action of sucrose on the oxidative enzymes.

Mitochondrial preparations from the leaves of other plants

A sedimentable preparation, which oxidized both succinate and malate, was obtained from the leaves of garden lupins (*Lupinus* sp.) by the usual procedure. The citrate in the extraction and washing media was essential. In one experiment 50 g. of leaves was divided into two equal lots; one lot was ground in the usual medium (70 ml.) and the other in the same medium without the citrate. A mitochondrial preparation from the first had a succinoxidase q_{O_2} (μl. of oxygen/mg. of protein N/30 min.) of 32 and an endogenous q_{O_2} of 5. That prepared from the second had an endogenous q_{O_2} of 4 and no succinoxidase. A sedimentable succinoxidase could also be prepared from the leaves of greenhouse-grown yams (*Dioscorea* sp.), but with this plant the citrate in the medium had no effect on the activity.

No succinoxidase activity could be prepared from the leaves of French beans (*Phaseolus vulgaris*).

DISCUSSION

The amount of phosphorylation catalysed by the tobacco-leaf preparations (30 μmoles of P/mg. of protein N/hr. in Fig. 1) could doubtless be increased by altering the assay conditions. Thus there was an indication that the phosphate concentration generally used, chosen for convenience of estimation, was not optimum. Nevertheless, the P/O ratios obtained are comparable with those from other green leaves (Smillie, 1956). They suggest that the mitochondria, or mitochondrial fragments, contained in these preparations retain some degree of organization. This conclusion is not at variance with the appearance of these preparations in some (unpublished) electron micrographs. However, the looseness of the connexion between the oxidative and phosphorylative reactions may indicate some functional damage. Rat-liver mitochondria contain a protein which is released on

Table 5. *Stability of oxidative and phosphorylative activities of mitochondrial preparations*

The mitochondrial preparation was divided into two lots before washing, and, after washing, one lot was suspended in water and the other in sucrose (0·25 M). Samples (0·5 ml.) were put into the side arms of manometer flasks, whose main compartments contained succinate with the medium described in the Experimental section, and were tipped into this medium after incubation for various times at 30°. Oxygen uptake was followed for 30 min. after it became regular (5 min.), and was corrected for endogenous activity. Phosphate disappearance was measured 40 min. after tipping and was not corrected for adenosine triphosphatase activity.

	Suspension in sucrose		Suspension in water	
Time of incubation (min.)	Oxidative activity (%)	Phosphorylative activity (%)	Oxidative activity (%)	Phosphorylative activity (%)
0	100	100	100	100
40	94	45	96	39
75	93	2	66	0

their disruption and which loosens the dependence of oxidations on phosphorylations in intact mitochondria (Remmert & Lehninger, 1959). Some similar process may have occurred during the grinding of the tobacco leaves. An alternative explanation, that the loose coupling represents the state of the mitochondria in the intact leaf, is possible but less likely.

The chloroplast material contaminating the mitochondrial preparations is unlikely to have any oxidative-phosphorylative activity (James & Das, 1957). Its removal would therefore increase the phosphorylative activity of the preparations, on a protein N basis, about threefold (Pierpoint, 1959). This activity increase, together with increases due to improvements in the method of estimation, may well bring phosphorylation rates to over 100 μmoles of P/mg. of protein N/hr., rates comparable with some of the highest of those reported for non-chlorophyllous plant material (see Allen, Whatley & Arnon, 1958). Whether they would ever reach the rates at which photosynthetic systems esterify phosphate (554–785 μmoles of P/mg. of protein N/hr. at 20°; Allen *et al.* 1958) is more difficult to foretell. Photosynthetic phosphorylation can supply the energy required for starch synthesis in tobacco-leaf disks (Maclachlan & Porter, 1959), and is probably the main source of ATP in the chlorophyllous cells that contain no mitochondria (James & Das, 1957; Weier, 1958). A comparison therefore of the maximal rates of photosynthetic and oxidative phosphorylation in extracts of the same leaves will give an idea of the relative contributions of the two energy-yielding systems in the intact leaf.

The usefulness of the citrate medium in the isolation of a sedimentable succinoxidase from lupin leaves is interesting in view of the results of Brummond & Burris (1954). Their experience with extracts of these leaves prepared in a sucrose solution lead them to question the location of the tricarboxylic acid-cycle complex of enzymes in the mitochondria of green leaves. It seems likely that a citrate- or succinate-containing medium would be helpful in the extraction of mitochondria from plants other than lupin and tobacco. Two recent papers suggest that this conclusion holds for a much wider range of tissues. One (Eichel, 1959) describes the stabilizing effect of citrate and succinate on some of the electron-transport enzymes of *Tetrahymena pyriformis*. The second deals with the increase in pyruvic oxidase activity of housefly mitochondria that results from including citrate, succinate and pyruvate in the extraction medium (Gregg, Heisler & Remmert, 1959). Ideas about the functioning of the insect mitochondria *in vivo* (Chance & Sacktor, 1958) are based on the properties of preparations made in the absence of these acids and may well need some revision.

SUMMARY

1. Further properties are described of a sedimentable preparation from tobacco leaves which has previously been shown to catalyse many of the oxidations of the tricarboxylic acid cycle.

2. The oxidation of some substrates was accompanied by an evolution of carbon dioxide which varied in amount between 0·3 and 1·5 times the volume of oxygen absorbed.

3. A disappearance of orthophosphate accompanied the oxidations provided that adenosine polyphosphates, glucose and hexokinase were present. The ratio of phosphate disappearing to oxygen absorbed (P/O ratio) was generally between 1 and 2. 2:4-Dinitrophenol completely supressed the phosphate disappearance and had less effect on oxygen uptake.

4. During the oxidation of substrate acids there was an increase in the amount of glucose 6-phosphate and nucleotide phosphate present. Incubation of the preparation with [^{32}P]orthophosphate resulted in radioactivity being incorporated into the nucleotide fraction. At least five times as much was incorporated in the presence as in the absence of oxidizable substrate.

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Mitochondrial Preparations from the Leaves of Tobacco (*Nicotiana tabacum*)

3. GLYCOLLIC OXIDASE AND FUMARASE ACTIVITY

BY W. S. PIERPOINT
Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts

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How far the cytochrome-linked oxidations of the mitochondrial preparations from tobacco leaves account for the oxygen uptake of intact leaves is a matter for speculation. Oxidases other than cytochrome oxidase are present in the leaves and may serve as 'terminal oxidases' in some conditions (James, 1953). Recent evidence has suggested that one of these, glycollic oxidase, is responsible for much of the uptake in detached leaves exposed to light (Zelitch, 1958). This has prompted these observations on the oxidation of glycollic acid by the leaf-mitochondrial extracts and the distribution of this activity among the other leaf fractions.

The observations on fumarase were expected to throw some light on the low rate of fumarate oxidation previously reported for these extracts (Pierpoint, 1959) and also on the integrity of the mitochondria. In animal tissues a considerable proportion of the fumarase is associated with mitochondria, but disruptive treatments liberate it in a non-sedimentable form (Kuff, 1954). The fumarase content of animal mitochondrial preparations is thus a useful indicator of mitochondrial damage (Mahler, Wittenberger & Brand, 1958). Much less is known about the location of plant fumarase, although active preparations have been made from pea-epicotyl mitochondria (Davies, 1956), and it has been detected in sedimented fractions of lupin leaves (Brummond & Burris, 1954).

EXPERIMENTAL

Oxygen uptake. Manometric conditions were the same as those described by Pierpoint (1960). Oxygen uptakes from glycollate (40 μmoles) and succinate (40 μmoles) were compared in the medium used to measure oxidative phosphorylation (final vol. 1·9–2·0 ml.), and oxygen uptake from fumarate was measured in the simpler medium used when only gas exchanges were being followed.

In one experiment oxidation of glycollate was also followed (Table 3) in a medium similar to that used by Zelitch (1957) for the estimation of glycollic oxidase. Its composition was: sodium glycollate, 20 μmoles; riboflavin 5′-phosphate, 0·1 μmole; 2-amino-2-hydroxymethylpropane-1:3-diol buffer (tris), pH 8·3, 0·3 m-mole; water to a final vol. of 2·1 ml. The enzyme activities measured in this manner were all within the region where activity was proportional to the amount of enzyme added.

Phosphorylation. The disappearance of orthophosphate was measured as previously described (Pierpoint, 1960). The P/O ratios quoted are only approximate, as no correction was made for phosphorylation during the 10 min. when the manometer flasks were equilibrating.

Fumarase activity. This was measured by a slight modification of the method of Racker (1950). The reaction mixture contained tris or phosphate buffer, pH 7·8 (0·15 m-mole), malate (0·15 m-mole), cysteine (2 μmoles) and enzyme preparation in a final volume of 3 ml. Increase in $E_{240\ m\mu}$ was measured at room temperature (21–25°) in a Unicam SP. 500 spectrophotometer, with the reaction mixture without malate as an optical blank. The reaction was followed for 20 min., although it proceeded linearly for at least 90 min.

A unit of fumarase was defined (Racker, 1950) as that which caused an increase in extinction of 0·001/min. Under the assay conditions, activity was proportional to added enzyme for at least 30 units.

The estimation of fumarase in the reverse direction, by observation of the disappearance of fumarate (0·05 m-mole) at 300 mμ, was only 5–7% as sensitive as the above method, mainly because of the relatively high absorption of the enzyme preparations at this wavelength.

Chemical estimations. Chlorophyll and protein N were estimated as described by Pierpoint (1960).

Chemicals. Tris and phosphate buffers were prepared as described by Pierpoint (1960).

RESULTS

Oxidation of glycollate by tobacco-leaf preparations

The mitochondrial preparations oxidized glycollate more quickly than any other organic acid so far tested. The rate was usually 130% of that of succinate under the same conditions, and only once was it less (87%). Oxygen uptake from a mixture of glycollate and succinate was greater than that from either substance alone, but less than their sum (86%). In contrast with this, tricarboxylic acid-cycle substrates did not augment the oxygen uptake due to succinate (Pierpoint, 1959). No phosphorylation accompanied the glycollate oxidation, and, in the presence of glycollate, phosphorylation from succinate was depressed by a quarter.

There are two other respects in which the oxidation of glycollate differed from that of succinate. It was not increased by the presence of cytochrome *c* in the incubation medium, and it was resistant to the presence of cyanide (Table 1). Bisulphite, an inhibitor of glycollic oxidase (Zelitch, 1957), inhibited it appreciably (Table 1), but also affected succinoxidase. It inhibited the phosphorylation from succinate more than the oxygen uptake, so that at a concentration of 4 μmoles, the P/O ratio was decreased by a quarter.

The presence of crystalline ox-liver catalase (27 μg./flask) did not decrease oxygen uptake from either glycollate or succinate.

The mitochondrial preparations oxidized lactate at a rate similar to that of succinate. This oxidation was inhibited by bisulphite to approximately the same degree as that of glycollate.

Table 1. *Differences between the oxidation of glycollate and succinate by mitochondrial fractions*

The mitochondrial preparations were incubated in the medium described in Pierpoint (1960), modified as below, and with either glycollate or succinate as substrate. The values for oxygen uptake, which are expressed as μl. of oxygen/mg. of protein N/30 min. (q_{O_2}), are corrected for the appropriate endogenous activities.

Expt. no.	Modification	Oxygen uptake in the presence of succinate (q_{O_2})	Oxygen uptake in the presence of glycollate (q_{O_2})
1	None	82	108
	Cytochrome *c* omitted	55	113
2	None	94	80
	Cyanide present (20 μmoles)	0	86
3	None	69	90
	Bisulphite present (2·4 μmoles)	50	20
	Bisulphite present (4 μmoles)	33	11

Fumarase activity of tobacco-leaf preparations

The mitochondrial preparations usually had a fumarase activity of about 1000 optical units/mg. of protein N. This is approximately 0·05% of the activity of crystalline pig-heart fumarase measured under slightly different conditions (Massey, 1951). A few properties of the activity were observed in the course of checking the method of estimation.

The pH optimum was between 7·8 and 8·0. Activity fell away steeply on either side of this region, and was down to about half at pH 7·2 and 8·5. There was no perceptible difference between the activities measured in phosphate buffer and those in tris buffer at the same pH. This was true even after the enzyme preparation had been washed in 0·25M-sucrose. But the traces of phosphate retained by the preparation may have been enough to mask a phosphate stimulation such as occurs with the fumarases of *Escherichia coli* (Mann & Woolf, 1930), pig heart (Massey, 1953) and yeast (Favelukes & Stoppani, 1958).

At lower substrate concentrations phosphate inhibited the fumarase. The inhibition varied with the concentration of phosphate and involved an alteration of the Michaelis constant (Fig. 1) suggesting that fumarate and phosphate competed for combination with some site on the enzyme. A similar competition occurs with yeast fumarase

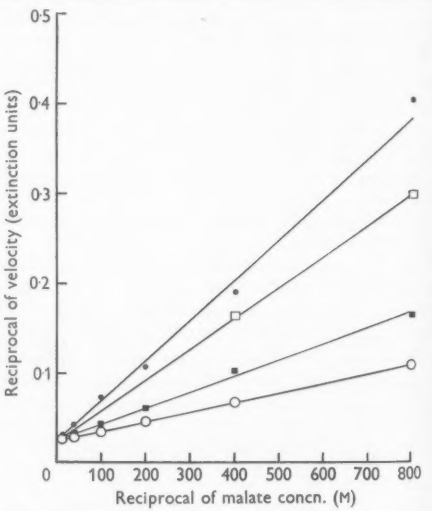

Fig. 1. Competitive inhibition of fumarase by phosphate. Enzyme activity at different substrate concentrations was measured in the presence of 0·05M-tris buffer (○), 0·05M-phosphate (●), 0·027M-phosphate (□) and 0·0067M-phosphate (■). The results are graphed by the method of Lineweaver & Burk (1934).

(Favelukes & Stoppani, 1958), although here it is complicated by a phosphate-stimulation effect. The Michaelis constant obtained for the tobacco-leaf enzyme in tris buffer differed a little from one preparation to another (2·2–4·2 mM), but was not much affected by changes in the concentration of tris.

Although added cysteine had only a small effect on its activity (0–10 %), the tobacco-leaf fumarase is probably SH-dependent, like the yeast enzyme (Favelukes & Stoppani, 1958). Both *p*-chloromercuribenzoate and iodoacetate inhibited the enzyme, and the inhibitions were partially reversed by cysteine. The reversal of the inhibition by *p*-chloromercuribenzoate was more complete and is shown in Table 2.

Table 2. *Effect of* p*-chloromercuribenzoate and cysteine on fumarase activity*

Samples (0·01 ml.) of a tobacco-leaf-mitochondrial preparation were incubated at room temperature with either phosphate buffer (pH 7·8, 0·2 m-mole) alone or buffer plus *p*-chloromercuribenzoate (PCMB; 0·15 μmole). After 20 min. malate (0·15 m-mole) was added and the extinctions (240 mμ) were measured every 3 min. Cysteine (1 μmole) was added to all tubes after 30 min. and again after 50 min.

	Fumarase activity (extinction units)	
	Without PCMB	With PCMB
Initial activity	11·5	1
After first cysteine addition	11·5	5
After second cysteine addition	11·5	7

Distribution of glycollic oxidase and fumarase among tobacco-leaf fractions

Although the mitochondrial preparations actively oxidized glycollate, their content of glycollic oxidase, measured by the method of Zelitch (1957), was less than one-tenth of the total in the extract (Table 3). The bulk of the activity (85 %) was present in the supernatant fraction, where it was twice as concentrated, relative to protein, as in the extract. No more than a trace of it (2–3 %) was sedimented on further centrifuging at 55 000 *g* for 2 hr.

In contrast, about 90 % of the fumarase of the disintegrated leaves was concentrated in the mitochondrial fraction, and most of the remainder was in the chloroplast fraction (Table 4). The high recovery of enzyme activity makes it unlikely that there was any enzyme activation or inhibition, and direct tests showed that the low fumarase activity in the supernatant fraction was not due to an inhibition by the extraction medium.

Treatments that would be expected to destroy mitochondrial structure liberated the fumarase in a

Table 3. *Distribution of glycollic oxidase among tobacco-leaf fractions*

Leaves (29 g.) were ground with sand (15 g.) in the usual medium (110 ml.), and the fractions sedimented at 1000 and 10 000 *g*. Each sedimented fraction was washed, and the washings of the mitochondrial fraction were added to the supernatant fraction. Chlorophyll, protein N and glycollic oxidase were measured in samples from each fraction, as well as in the initial extract. Endogenous oxygen uptake was subtracted from the uptake in the presence of glycollate in calculating the glycollic oxidase activity.

	Protein N		Chlorophyll		Glycollic oxidase	
Fraction	mg.	% of total	mg.	% of total	μl. of oxygen uptake/30 min./ mg. of protein N	% of total
Initial extract	80	100	31·6	100	170	100
Chloroplast	11·5	15	12	39	21	1·8
Mitochondrial	8·9	11	9·2	29	110	7·5
Supernatant	33	42	1·25	4	340	85

Table 4. *Distribution of fumarase among tobacco-leaf fractions*

Leaves (29 g.) were ground with sand (15 g.) in the usual medium (150 ml.), and the fractions sedimented at 1000 and 10 000 *g*. Each sedimented fraction was washed, the washings of the chloroplast fraction being added to the supernatant from which the mitochondrial fraction was sedimented, and the washings of this fraction to the final supernatant. Chlorophyll, protein N and fumarase were estimated in all fractions.

	Protein N		Chlorophyll		Fumarase		
Fraction	mg.	% of total	mg.	% of total	Units	% of total	Units/mg. of protein N
Initial extract	80	100	24·7	100	9600	100	120
Chloroplast	14	18	15·2	62	535	6	40
Mitochondrial	8	10	6·4	26	8000	85	1000
Supernatant	50	64	0·8	3·2	<300	<3	<6

less sedimentable form. In particular, three-quarters of the fumarase in a preparation that had been kept at 37° for 2 hr. and then frozen and thawed three times remained in the supernatant after centrifuging at 50 000 *g* for 2½ hr.

Fumarate oxidation by mitochondrial preparations

The rate of fumarate oxidation by mitochondrial preparations was about 2·8 μg.atoms of oxygen taken up/mg. of protein N/30 min. that is between a third and a half of the rate of malate or succinate oxidation. This is an improvement over the rates previously obtained (Pierpoint, 1959), possibly because more extraction medium was used, but it still suggests that the rate-limiting step in the oxidation is in the conversion of fumarate into malate.

There was, however, no evidence that the rate of fumarate oxidation was limited by the fumarase activity of the preparations. In the experiment described in Table 4, the mitochondrial fraction catalysed an oxygen uptake from fumarate of 1·8 μg.atoms/mg. of protein N/30 min. The fumarase of this preparation, however, catalysed the dehydration of 46 μmoles of malate/mg. of protein N/30 min. in the spectrophotometric estimation. This value was only decreased to 28 μmoles when the estimation was made more comparable with the manometric oxidation by changing the pH to 7·3, omitting the cysteine and decreasing the malate concentration. As the fumaric oxidase was estimated at 30° and fumarase at room temperature, this 15-fold difference between their activities is a minimal value.

DISCUSSION

Oxidation of glycollate

The oxidation of glycollate by the mitochondrial preparations differed from the oxidation of tricarboxylic acid substrates. It was not much affected by the presence of succinate and was not associated with phosphorylation reactions. The most likely mechanism for the oxidation is:

$$HO{\cdot}CH_2{\cdot}CO_2H + O_2 \rightarrow CHO{\cdot}CO_2H + H_2O_2$$

catalysed by the bisulphite-sensitive glycollic oxidase (Kenten & Mann, 1952; Zelitch, 1957). The mitochondrial preparations contained sufficient catalase activity to decompose rapidly the hydrogen peroxide formed, so that the added catalase did not lower oxygen uptake as in the experiments of Kenten & Mann (1952).

Zelitch (1958) has demonstrated a large accumulation of glycollate in illuminated tobacco leaves when they are exposed to either bisulphite or bisulphite–aldehyde addition compounds. On the basis of the specific inhibition of glycollic oxidase by these substances (Zelitch, 1957), he suggested an important role for this enzyme in the respiration of leaves in sunlight. Bisulphite was the least specific of these inhibitors, but its inhibition of tobacco-leaf succinoxidase (Table 1) raises the possibility that, in the experiments of Zelitch, this substance at least may have prevented the oxidation of glycollate by a cytochrome system. Although no evidence for such a cytochrome-dependent oxidation was observed in the present work, the argument for a respiratory function of glycollic oxidase would be strengthened if the aldehyde bisulphite compounds were shown not to affect the cytochrome oxidations carried out by the tobacco-leaf-mitochondrial preparations.

Intracellular location of fumarase and glycollic oxidase

In rat liver about half the fumarase is located in the mitochondria, from which it is released by sonic disruption (Kuff, 1954; de Duve, Pressman, Gianetto, Wattiaux & Applemans, 1955). The rest is distributed between smaller sedimentable particles and the supernatant fraction. The intracellular location of the extramitochondrial enzyme is in doubt, but there is some reason for believing that its presence on the small sedimentable particles is due to adsorption during cell disruption (Kuff, 1954).

Virtually all the tobacco-leaf fumarase is present in the mitochondrial fraction, where its activity per mg. of protein N is greater than in any other fraction. There was no reason to believe that any activation or inhibition of the enzyme occurred in any fraction, so it is reasonable to conclude (Alexander & Wilson, 1955) that in the intact cell it is attached to some component of the fraction other than the chlorophyll-containing fragments. It is likely that some, or even most, of it is attached to the same components that carry the enzymes of the tricarboxylic acid cycle; its release from this fraction by gentle disruptive treatments supports this view. But some of it may well be associated with other constituents of this fraction. Of importance in this context is the fate of the nucleic acid-containing particles of tobacco leaves (Pirie, 1950) in extraction media with high salt content. They may well have sedimented with the mitochondria, bearing any fumarase from the supernatant fluid with them. If this were so, sedimentable differences between the leaf and the animal fumarases may be more apparent than real.

Tolbert & Cohan (1953) suggested that about a tenth of the total glycollic oxidase of barley leaves is associated with chloroplasts. The difficulty about conclusions concerning such small proportions of a total activity is, however, well recognized (Alexander & Wilson, 1955). There is no alternative

but to regard the glycollic oxidase of the sedimentable fractions of tobacco leaves as a contaminant from the supernatant fraction. If the enzyme is associated with any microscopic structure of the tobacco cells, the linkage is labile and is completely disrupted during the disintegration of the leaves. Such a linkage would be quite unlike that holding fumarase on to the mitochondria.

SUMMARY

1. Preparations from tobacco leaves, which sedimented between 1000 and 10 000 *g*, oxidized glycollate and lactate. The oxidations did not appear to involve cytochromes and were probably brought about by glycollic oxidase.

2. The glycollic oxidase of these preparations was only a small (7·5 %) proportion of the total in the leaf extract. The bulk of the activity was in the supernatant fraction and was not sedimented by centrifuging at 55 000 *g* for 2 hr.

3. In contrast, the sedimentable preparations contained nearly all (85–92 %) of the fumarase in the extracts. Ageing at 30°, followed by freezing and thawing, liberated three-quarters of this activity in a form not sedimented at 50 000 *g*.

4. The fumarase was inhibited by *p*-chloromercuribenzoate and iodobenzoate and partially reactivated by cysteine. It was competitively inhibited by phosphate.

5. The fumarase activity was in excess of that required for its expected role in the oxidation of fumarate by the preparations.

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Carbon Assimilation by *Pseudomonas oxalaticus* (OX1)

3. OXALATE UTILIZATION DURING GROWTH ON OXALATE*

BY J. R. QUAYLE AND D. B. KEECH†
Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford

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It has been shown (Quayle & Keech, 1959*a*) that one of the key enzymes of the ribulose 1:5-diphosphate cycle of carbon dioxide fixation (Bassham *et al.* 1954), carboxydismutase, is not synthesized when *Pseudomonas oxalaticus* (OX1) is grown on oxalate. This indicates that such a cycle cannot be of major importance in the conversion of oxalate carbon atoms into cellular material in oxalate-grown cells, in contrast with formate-grown cells of the same organism (Quayle & Keech, 1959*b*, *c*). The present work, by giving information on the activities of phosphoribulokinase and phosphoriboisomerase during adaptation of oxalate-grown cells to growth on formate, confirms this conclusion. The sequence of compounds which become labelled when the organism is growing on [^{14}C]-oxalate has also been determined, and a route for incorporation of oxalate into cellular constituents, based on these results and on reactions observed in

* Part 2: Quayle & Keech (1959*c*).

† Present address: Department of Biochemistry, Western Reserve University, Cleveland, Ohio, U.S.A.

crude cell-free extracts, is proposed. A preliminary account of part of this work has been published (Quayle & Keech, 1959*d*).

METHODS AND MATERIALS

Maintenance and growth of the organism. The maintenance and growth of cultures of *Pseudomonas oxalaticus* (OX1) have been described (Quayle & Keech, 1959*b*), sodium oxalate being used in place of sodium formate in the liquid media. Large quantities of the organism were grown in 10 l. of medium inoculated with 400 ml. of a bacterial suspension in the logarithmic phase of growth. The growth vessel used was a glass bottle vigorously aerated by blowing in air through a pumice plug fixed to the end of a submerged inlet tube. Growth was maintained at 30°, oxalic acid (A.R.) being added to keep the pH at 7·5–8·5. After 48 hr. the bacteria (15 g. wet wt.) were harvested in a Sharples centrifuge and stored at −10°.

Manometric methods and preparation of tracer solutions. The procedures used have been described previously (Quayle & Keech, 1959*b*). Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks.

Bacterial incorporation of [^{14}C]*oxalate,* [^{14}C]*formate and* [^{14}C]*bicarbonate.* Bacteria which had been grown in conical (Fernbach) culture flasks were centrifuged at room temperature and resuspended in a small volume of their supernatant medium. A sample (1 ml.) of this suspension, containing from 13 to 24 mg. dry wt. of bacteria, was added either to fresh growth medium (8 ml.) containing 100 mM-sodium oxalate or to 10 mM-potassium phosphate, pH 7·0 (8 ml.), containing 10 mM-sodium oxalate (in the [^{14}C]oxalate experiment) in a cylindrical vessel (10 cm. × 2·5 cm. diam.) fitted with a sintered-glass bottom. Vigorous aeration in the suspension was maintained by blowing a stream of air through the sintered glass. Incubation was continued for 5 min. (or 1 hr. in the [^{14}C]bicarbonate experiment) to ensure that growth had recommenced.

In the [^{14}C]bicarbonate experiment fresh oxalic acid was continually added to keep the pH at 7·5–8·5. At the end of the preincubation period, 200 μc of ^{14}C-tracer solution was added to bring the final volume of the suspension to 10 ml. Samples (1 ml.) were withdrawn with a hypodermic syringe at suitable time intervals over 10 min. and transferred immediately to 3 ml. of boiling aqueous 95% (v/v) ethanol.

In the [^{14}C]formate experiment a parallel manometric measurement of the extent of formate oxidation was made. A sample (2 ml.) of a similarly prepared bacterial suspension was placed in the main compartment of a double-side-arm manometer cup, which contained 400 μmoles of CO_2-free sodium hydroxide in the centre well. The two side arms contained respectively 50 μc of [^{14}C]formate in 0·4 ml. of water and 3·3 m-moles of sulphuric acid. The sulphuric acid was tipped in to terminate the reaction and the manometer was then shaken for 1 hr. to ensure that all the liberated $^{14}CO_2$ was trapped in the centre well, whose contents were then transferred with washing to a stoppered tube and made to a total volume of 2 ml. with water. The radioactivity in this solution was assayed as barium [^{14}C]carbonate.

Treatment of aqueous ethanolic suspensions of cells. The hot ethanolic suspensions described previously were centrifuged and the resulting precipitates extracted with 1 ml. of hot aqueous 20% (v/v) ethanol. The combined supernatant fractions were evaporated to dryness at 60° under N_2. Those resulting from the [^{14}C]formate and [^{14}C]bicarbonate experiments were redissolved in 0·25 ml. of aqueous 20% (v/v) ethanol ready for application to the origin position of paper chromatograms. Those fractions resulting from the [^{14}C]oxalate experiments were freed from residual [^{14}C]oxalate by incubation in the following manner with purified oxalate decarboxylase, prepared from *Collybia velutipes* by the method of Shimazono & Hayaishi (1957). The fractions were redissolved in 0·5 ml. of water and treated with a small quantity of Dowex-50 (H^+ form) to remove cations which inhibited the activity of the decarboxylase. The resin was removed by centrifuging, washed with 0·2 ml. of water and the combined supernatant fraction adjusted to pH 4·5 with 0·1 M-acetate buffer, pH 4·5. A portion (0·1 ml.) of an aqueous solution of purified oxalate decarboxylase was added and the fractions were incubated at room temperature for 5 hr.; 1 μmole of oxalic acid and a further 0·1 ml. of decarboxylase were then added and incubated for a further 3 hr. Samples (0·5 ml.) of the resulting solutions were applied to the origin position of paper chromatograms.

Chromatographic analysis and radioautography. The procedures used have been described previously (Quayle & Keech, 1959*b*).

Identification of labelled compounds. The radioactive compounds were eluted from the chromatogram with water and chromatographed in appropriate solvent systems together with authentic samples of the suspected compound. Exact matching of the resulting radioactive areas and the coloured area developed with an appropriate spray reagent was necessary for identification. Carboxylic acids were rechromatographed either in the original solvent systems or in ethanol–aq. NH_3 soln. (sp.gr. 0·880)–water (80:4:16, by vol.; Long, Quayle & Stedman, 1951) for 8 hr. Amino acids were rechromatographed in water-saturated phenol in the presence of NH_3 vapour (Consden, Gordon & Martin, 1944) for 16 hr. or in butanol–acetone–water–diethylamine (10:10:5:2, by vol.; Hardy, Holland & Nayler, 1955) for 8 hr. The last-named solvent was found particularly useful for separating serine, glycine and glutamate. Dephosphorylation of phosphorylated compounds was carried out by incubation of the eluted compounds with a phosphatase fraction, obtained from Polidase-S (Schwarz Laboratories Inc., New York, U.S.A.), by precipitation in 85–100% saturated aqueous ammonium sulphate. The resulting compounds were chromatographed two-dimensionally for 12 hr. in water-saturated phenol and butanol–propionic acid–water (47:22:31, by vol.; Benson *et al.* 1950). The sprays used for locating various compounds on chromatograms were those described by Quayle & Keech (1959*b*).

Preparation of bacterial extracts for enzyme assay. Samples of bacteria were obtained from culture solutions by centrifuging. The resulting bacterial samples, containing 30–50 mg. dry wt. of bacteria, were each suspended in 1 ml. of mM-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·8, containing about 200 μg. each of crystalline ribonuclease and deoxyribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks). Extracts were then prepared by disrupting the bacteria with a Mullard–MSE ultrasonic cell disintegrator (19 kcyc. at 75 w) for 7 min. at 0°, followed by centrifuging at 25 000 *g* for 20 min. in a refrigerated centrifuge.

Carboxydismutase assay. A sample (0·05 ml.) of extract, containing about 1 mg. of total protein, was incubated with 5 μmoles of tris buffer, pH 7·8, 2·5 μmoles of magnesium chloride, 2·5 μmoles of reduced glutathione (GSH), 0·2 μmole of ribulose 1:5-diphosphate, 0·45 μmole of sodium bicarbonate (10 μc of ^{14}C) and water to 0·35 ml. for 15 min. at 30°. The reaction was stopped with 1 ml. of hot ethanol and samples (0·1 ml.) were plated in a stream of warm air on glass planchets mounted on a rotating turn table. The plates were irrigated with 0·2 ml. of formic acid (90%, w/v; A.R.), dried and the radioactivity on them was assayed with an end-window, lead-shielded Geiger–Müller tube (General Electric Co. Ltd.). Carboxydismutase activity is expressed as total radioactivity incorporated (counts/min.)/μg. of bacterial protein. Chromatographic analysis and radioautography of the soluble radioactive components showed 3-phosphoglyceric acid (PGA) to contain over 85% of the total radioactivity.

Phosphoriboisomerase assay. The method of Hurwitz, Weissbach, Horecker & Smyrniotis (1956) was used, in which the ribulose 5-phosphate formed from ribose 5-phosphate is estimated by the cysteine–carbazole colorimetric method. One unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of 0·6 μmole of ribulose 5-phosphate in 10 min. under the experimental conditions.

Phosphoribulokinase assay. The enzyme was assayed by measuring [^{14}C]3-phosphoglyceric acid formation from ribose 5-phosphate, adenosine triphosphate (ATP) and sodium [^{14}C]bicarbonate as a result of the following reactions:

$$\text{Ribose 5-phosphate} \rightarrow \text{ribulose 5-phosphate} \quad (1)$$

$$\text{Ribulose 5-phosphate} + \text{ATP} \rightarrow \text{ribulose 1:5-diphosphate} + \text{ADP} \quad (2)$$

$$\text{Ribulose 1:5-diphosphate} + [^{14}C]\text{bicarbonate} \rightarrow 2[^{14}C]\text{PGA} \quad (3)$$

Purified carboxydismutase, catalysing reaction (3), was prepared from spinach by the method of Weissbach, Horecker & Hurwitz (1956) and a portion was added to each sample in excess. Phosphoriboisomerase, catalysing reaction (1), was found to be present in all the samples. Under these conditions, phosphoribulokinase, catalysing reaction (2), was the limiting step. The complete assay mixture contained 5 μmoles of tris buffer (pH 7·8), 2·5 μmoles of magnesium chloride, 2·5 μmoles of GSH, 2 μmoles of ribose 5-phosphate, 5 μmoles of ATP, 0·45 μmole of sodium bicarbonate (10 μc of ^{14}C), 0·2 ml. of carboxydismutase solution and 0·05 ml. of cell-free extract to be assayed. The mixture was incubated for 15 min. at 30° and the reaction was stopped with 1 ml. of hot ethanol and samples (0·1 ml.) were assayed on glass planchets for radioactivity as previously described.

Special materials. Acetyl-coenzyme A was a gift of Dr G. H. Dixon. A culture of *Collybia velutipes* was a gift of Mr Kemp, Department of Botany, University of Oxford.

Protein determinations. Protein was estimated by the method of Folin & Ciocalteu (1927).

RESULTS

Oxidation of formate and oxalate. Washed suspensions of *P. oxalaticus* oxidized both formate and oxalate with Q_{O_2} values ranging from -40 to -150 (μl. of O_2 consumed/mg. dry wt. of bacteria/hr.), depending on the condition of the culture harvested.

Incorporation of [^{14}C]*formate and* [^{14}C]*bicarbonate.* The operation of an autotrophic type of metabolism similar to that found in the formate-grown organism, during growth on oxalate should lead to a major incorporation of carbon dioxide by the cells. If in such a case the breakdown of oxalate proceeded via formate, then a major incorporation of formate carbon might also be expected. In order to test these possibilities the incorporation of either [^{14}C]formate or [^{14}C]bicarbonate into cells growing on oxalate was measured. In both cases negligibly small incorporation of isotope into the ethanol-soluble fraction of the cells was observed. With [^{14}C]bicarbonate the only compound that appeared labelled after incubation for 10 min. was malic acid. With [^{14}C]formate, a parallel determination was carried out in a Warburg manometer cup of the extent of [^{14}C]formate oxidation that had occurred during the incubation. It was found that the radioactivity of the [^{14}C]carbonate in the centre well accounted for 31% of the radioactivity added as [^{14}C]formate at the beginning of the experiment, showing that formate was being metabolized by the cells and not being excluded from them by permeability barriers. These results contrast with the incorporation of [^{14}C]oxalate (see later), in which over 30 labelled compounds were obtained after incubation for 2 min., and show that neither formate nor carbon dioxide lies on the main pathway of carbon incorporation from oxalate, unless exogenous formate and carbon dioxide do not mix with endogenous formate or carbon dioxide formed from oxalate. Such a situation might exist because of compartment barriers within the cell or the utilization of the metabolites in an activated form.

The results also show that different metabolic pathways must operate during growth on either formate or oxalate, the autotrophic type of metabolism found with the former growth substrate being replaced in the latter substrate by some process not involving a major incorporation of formate carbon or carbon dioxide.

This change in metabolism with the change of growth substrate was checked by following the changes in activities of carboxydismutase, phosphoribulokinase and phosphoriboisomerase during adaptation of the cells to growth on either oxalate or formate. These three enzymes are necessary for the operation of the ribulose 1:5-diphosphate cycle of carbon dioxide fixation.

Enzymic changes during adaptation of the oxalate-grown organism to growth on formate. *P. oxalaticus* was grown in 2·2 l. of oxalate growth medium at 30° to a density of 0·15 mg. dry wt. of bacteria/ml. and centrifuged. The centrifuged cells were resuspended to a final vol. of 15 ml. in aqueous 0·9 % potassium chloride; 2 ml. was retained for enzyme assay and the remaining 13 ml. resuspended in 5·5 l. of formate growth medium contained in a 10 l. glass jar. Aeration was maintained through a plug of pumice sealed at the end of a submerged glass inlet tube, the temperature being maintained at 30°. Growth was measured nephelometrically and the pH was maintained at 7·0–8·5 by addition of formic acid (90 %, w/v; A.R.); samples (750 ml.) were withdrawn at suitable time intervals and the bacteria collected from them by centrifuging. The bacterial samples were assayed for carboxydismutase, phosphoribulokinase and pentose phosphate-isomerase activities. The oxalate-grown cells had negligibly low activities of carboxydismutase and phosphoribulokinase (Fig. 1); these activities rose sharply in the lag period of no growth (12 hr.) and were then maintained during growth on formate. The rise in carboxydismutase activity preceded that of the kinase. A high pentose phosphate-isomerase activity was found in both the oxalate-grown cells and formate adapted cells.

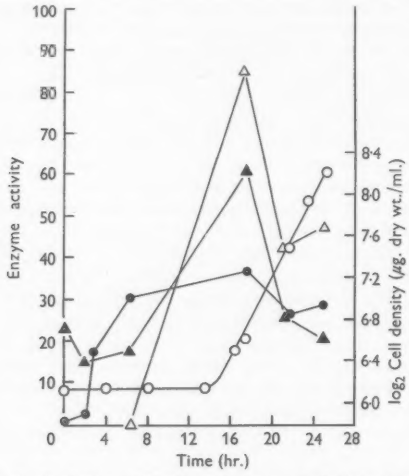


Fig. 1. Variation with time of enzyme activities during adaptation of oxalate-grown *P. oxalaticus* to growth on formate. ○, Growth of organism; ●, carboxydismutase activity; expressed as counts/min./μg. of protein; △, phosphoribulokinase activity, expressed as counts/min./mg. of protein; ▲, 10^{-1} × pentose phosphate-isomerase activity, expressed as units/mg. of protein.

The effect of the presence in the growth medium of chloramphenicol on the appearance of carboxydismutase activity during the lag period was tested. Oxalate-grown *P. oxalaticus* was suspended in 5·6 l. of formate growth medium at a cell density of 0·15 mg. dry wt./ml. and the resulting suspension was divided between two 10 l. growth vessels, which were aerated at 30°. After 2·25 hr. chloramphenicol was added to one flask to a final concentration of 50 μg./ml. Samples (350 ml.) of the two cultures were harvested at time intervals and tested for carboxydismutase activity. No rise in enzyme activity was observed in the flask to which chloramphenicol had been added (Fig. 2), suggesting that the appearance of carboxydismutase activity represents an actual synthesis of new enzyme protein rather than an unmasking of enzyme activity.

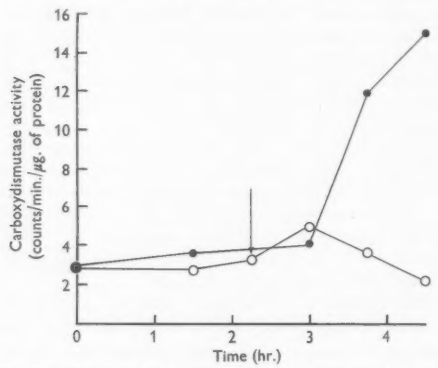


Fig. 2. Influence of chloramphenicol on the appearance of carboxydismutase activity in oxalate-grown cells suspended in formate growth medium. ●, Control suspension; ○, chloramphenicol added to final concn. of 50 μg./ml. at the time indicated by the arrow.

Enzymic changes during adaptation of the formate-grown organism to growth on oxalate. *P. oxalaticus* was grown in 1·6 l. of formate growth medium at 30° to a density of 0·17 mg. dry wt. of bacteria/ml. and centrifuged. The centrifuged cells were resuspended to a final vol. of 15 ml. in aqueous 0·9 % potassium chloride; 2 ml. was retained for enzyme assay and the remaining 13 ml. resuspended in 2 l. of oxalate growth medium contained in a 2·5 l. bottle. Growth was maintained as described previously under the reverse adaptation experiment, oxalic acid (A.R.) being added to maintain the pH at 7·0–8·5. When the bacterial culture had doubled in cell density, 1 l. was harvested and 1 l. of fresh oxalate growth medium was returned to the culture vessel. This was repeated through seven bacterial generations. During the first generation of growth the carboxydismutase activity was maintained almost unchanged; thereafter decrease in

activity closely followed the theoretical diluting-out to be expected if the enzyme was no longer being synthesized (Fig. 3). After seven generations of growth in a constant concentration of oxalate, formate was added to a concentration one-tenth that of the oxalate. A rapid increase in carboxydismutase activity was observed without a detectable change in growth rate.

Attempted induction of carboxydismutase synthesis under different growth conditions. The following experiment was undertaken to determine whether the stimulation of carboxydismutase synthesis after the addition of formate, as described in the previous experiment, could be repeated under different growth conditions. Starting from inocula taken from oxalate–agar slopes, separate cultures of organisms were grown in oxalate, lactate and succinate liquid growth media (acetate and ribose were also tried, but the organism failed to grow on these substrates). When the cultures had reached a suitable cell density, sodium formate was added to the growth media to a final concentration of 10 mM. Samples of the bacteria were harvested by centrifuging during the 2 hr. period subsequent to the formate addition and tested for carboxydismutase activity in the manner previously described. In no case was any appreciable carboxydismutase activity observed.

It has previously been observed by Fuller (1956) and Fuller & Gibbs (1959) that a fivefold increase of carboxydismutase activity could be induced in xylose- or arabinose-grown *Escherichia coli* (Crookes strain) by passing air + CO_2 (95:5, v/v) through the

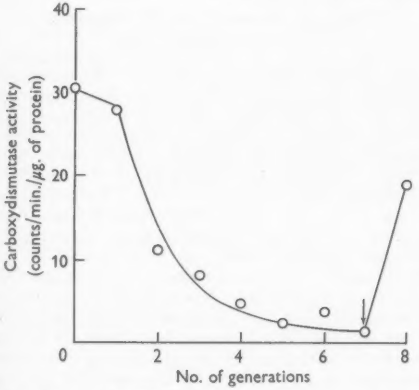


Fig. 3. Change of carboxydismutase activity in formate-grown *P. oxalaticus* plotted against the number of generations grown in oxalate medium after adaptation. 10 mM-Formate was added after the seventh generation was harvested.

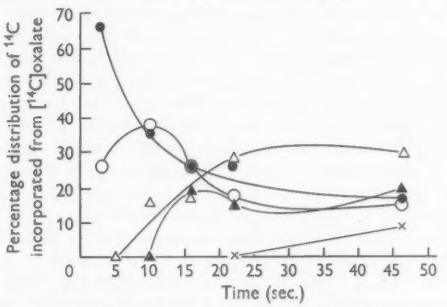


Fig. 4. Variation with time of the percentage distribution of ^{14}C from [^{14}C]oxalate incorporated into the constituents of the ethanol-soluble fraction of *Pseudomonas oxalaticus* growing on oxalate. Conditions were as described in Table 1. ○, Phosphoglycerate; ●, glycine; △, phosphates of glucose, fructose and sedoheptulose; ▲, alanine; ×, glutamate.

Table 1. *Distribution of radioactive carbon amongst the non-volatile labelled components of the ethanol-soluble fractions of growing suspensions of* Pseudomonas oxalaticus *incubated with sodium* [^{14}C]*oxalate*

Sodium [^{14}C]oxalate (10 μmoles; 200 μc of ^{14}C) was added at zero time to 10 ml. of a suspension of bacteria (20 mg. dry wt.) vigorously aerated in 10 mM-sodium oxalate, 10 mM-potassium phosphate, pH 7·0. Samples (1 ml.) were withdrawn by hypodermic syringe at known times and transferred into 3 ml. of hot ethanol. The precipitates were each extracted with 1 ml. of hot aqueous 20% (v/v) ethanol and the extracts were analysed by two-dimensional chromatography. The radioactive areas were located by radioautography and directly counted with a mica end-window Geiger–Müller tube.

	Radioactivity (counts/min.)								
Time (sec.)	Phospho-glyceric acid	Glucose phos-phates	Fructose phos-phates	Sedohep-tulose phos-phates	Glycine	Glutamate	Alanine	Other compounds	Total
3	136	0	0	0	340	0	0	38	514
10	224	36	41	20	206	0	0	67	594
16	243	75	51	30	237	0	172	118	926
22	162	168	64	30	265	0	135	103	927
46	250	300	138	50	268	130	328	237	1701

bacterial suspension, which suggested that carbon dioxide partial pressure may affect carboxydismutase synthesis. However, no significant change in carboxydismutase activity in *P. oxalaticus* growing on oxalate was found when the bacterial suspension was aerated with air + CO_2 (95:5, v/v) instead of air alone, even when the cells had adapted from growth on formate.

A comparison between the effect of addition of formate to the growth medium and aeration with air + CO_2 (95:5, v/v) upon the carboxydismutase activity in pentose-grown *E. coli* was made to see whether formate had the same effect as increased CO_2 partial pressure as noted by Fuller & Gibbs. However, no appreciable carboxydismutase activity was observed under either of the two growth conditions with ribose-grown *E. coli* (NCIB no. 8571).

Incorporation of [^{14}C]*oxalate into cells growing on oxalate.* A growing suspension of *P. oxalaticus* was incubated with [^{14}C]oxalate and the course of incorporation of the ^{14}C into the cells was followed by chromatographic and radioautographic techniques. After incubation for 2 min. over 30 labelled compounds were found in the aqueous ethanol-soluble fractions of the cells. The distribution of radioactivity amongst the labelled compounds formed during the first 46 sec. of incubation is shown in Table 1. The curves obtained by plotting the percentage of the total radioactivity contributed by each constituent of the sample against time of incubation are shown in Fig. 4. Radioactive malate was also formed but it has been included under the heading 'Other compounds' in Table 1, owing to the presence of traces of a radioactive impurity in the [^{14}C]oxalate. This impurity chromatographed together with authentic malate in all solvents tried and masked the time at which [^{14}C]malate was formed in the cells. The only labelled compounds formed in 3 sec. and isolated under these conditions were glycine and PGA. During the incubation period of 3–10 sec., the percentage of radioactivity contained in glycine diminished while that in PGA increased. This shows that isotope from [^{14}C]oxalate enters glycine more rapidly than PGA. Other metabolites such as sugar phosphates, alanine and glutamate become labelled later. The following work with cell-free extracts indicates the possible nature of these initial transformations.

Formation of glycerate in cell-free extracts

Formation of glycerate from glyoxylate. Crude cell-free extracts prepared from the organism catalyse a rapid anaerobic decarboxylation of glyoxylate in which 1 mole of CO_2 is formed from 2 moles of glyoxylate, a reaction similar to that discovered in *E. coli* by Krakow & Barkulis (1956). These workers suggested a C_3 compound such as hydroxypyruvate or tartronic acid semialdehyde as being the reaction product:

$$2\ \begin{matrix}\mathrm{CHO}\\ |\\ \mathrm{CO_2H}\end{matrix} \rightarrow \left[\begin{matrix}\mathrm{CH_2{\cdot}OH} & \mathrm{CHO}\\ | & |\\ \mathrm{CO} & \rightleftarrows\ \mathrm{CH{\cdot}OH}\\ | & |\\ \mathrm{CO_2H} & \mathrm{CO_2H}\end{matrix}\right] + \mathrm{CO_2} \qquad (1)$$

When [1-^{14}C]glyoxylate was incubated anaerobically with an extract prepared from the cells by disruption in a Hughes's (1951) press, the formation of radioactive glycerate was found (Table 2). The yield of glycerate was increased by the addition of reduced diphosphopyridine nucleotide (DPNH).

These reactions are similar to those found in glycollate-grown *Pseudomonas ovalis* Chester by

Table 2. *Formation of* [^{14}C]*glycerate from* [1-^{14}C]*glyoxylate*

The complete system contained, in a Thunberg tube: 100 μmoles of potassium phosphate–sodium phosphate, pH 6·9; 20 μmoles of $MgCl_2$; 10 μmoles of GSH; 2 μmoles of DPNH; 1·2 μmoles of sodium [1-^{14}C]glyoxylate (0·25 μc); 0·25 ml. of bacterial extract and water to 2·4 ml. [The extract was prepared by disrupting 1 g. of bacteria in 3 ml. of phosphate buffer (0·03 M; pH 6·9) in a Hughes's (1951) press cooled to −25°; the resulting extract was centrifuged at 25 000 *g* for 20 min. and the residue discarded.] The evacuated Thunberg tubes were incubated at 30° for 15 min. and the reaction was stopped by freezing the tubes in liquid air, opening them and adding 8 ml. of absolute ethanol. After the tubes had thawed, the precipitated protein was centrifuged, washed with 0·2 ml. of aqueous ethanol (20%, v/v) and the combined supernatants were evaporated at 30° under reduced pressure of N_2. The products were analysed by chromatography and radioautography.

Contents of tubes	Radioactivity (counts/min.) in glycerate
Complete system	5650
DPNH omitted	2380
Boiled extract used	0

Table 3. *Formation of* [^{14}C]*glycerate from* [^{14}C]*oxalate*

Experimental conditions were the same as those described in Table 2. The complete system contained, in a Thunberg tube: 100 μmoles of potassium phosphate–sodium phosphate, pH 6·9; 20 μmoles of $MgCl_2$; 10 μmoles of GSH; 2 μmoles of DPNH; 0·25 μmole of acetyl-CoA; 2 μmoles of sodium [^{14}C]oxalate (1·25 μc); 0·25 ml. of bacterial extract; water to 2·4 ml.

Contents of tubes	Radioactivity (counts/min.) in glycerate
Complete system	710
DPNH omitted	60
Acetyl-CoA omitted	159
DPNH and acetyl-CoA omitted	60
Boiled extract used	0

Kornberg & Gotto (1959), and postulated in a pseudomonad grown on glycine (Callely & Dagley, 1959), and demonstrate a reductive conversion of glyoxylate into glycerate.

Formation of glycerate from oxalate. Formation of [^{14}C]glycerate from [^{14}C]oxalate was also observed in these extracts in the presence of acetyl-coenzyme A (acetyl-CoA) and DPNH (Table 3). No other product was found on the chromatograms and addition of these cofactors separately resulted in little synthesis of glycerate. These results indicate that the following reactions may occur in the crude extract under the test conditions:

$$2\ \text{Oxalate} + 2\ \text{acetyl-CoA} \rightarrow 2\ \text{oxalyl-CoA} + 2\ \text{acetate} \quad (2)$$

$$2\ \text{Oxalyl-CoA} + 2\ \text{DPNH} \rightarrow 2\ \text{glyoxylate} + 2\ \text{CoA} + 2\ \text{DPN} \quad (3)$$

$$2\ \text{Glyoxylate} + \text{DPNH} \rightarrow \text{glycerate} + \text{CO}_2 + \text{DPN} \quad (4)$$

Reaction (2), involving an acetyl-CoA transphorase, would lead to oxalyl-CoA, evidence for the existence of which has previously been presented for a bacterium growing in the presence of oxalate (Jakoby, Ohmura & Hayaishi, 1956). The reduction of an acyl-CoA grouping to an aldehyde grouping, as suggested in (3), has already been demonstrated (Burton & Stadtman, 1953) in extracts of *Clostridium kluyveri* with acetyl-CoA:

$$\text{Acetyl-CoA} + \text{DPNH} \rightleftarrows \text{acetaldehyde} + \text{CoA} + \text{DPN} \quad (5)$$

The over-all effect of reactions (2)–(4) would be to condense oxalate reductively to glycerate and CO_2:

$$2\ \text{Oxalate} + 2\ \text{acetyl-CoA} + 3\ \text{DPNH} \rightarrow \text{glycerate} + 2\ \text{acetate} + 2\ \text{CoA} + \text{CO}_2 + 3\ \text{DPN} \quad (6)$$

DISCUSSION

The changes in enzyme activity observed when *P. oxalaticus* adapts from growth on formate to growth on oxalate, and vice versa, show that the ribulose 1:5-diphosphate cycle of carbon dioxide fixation is not used to any major extent during growth on oxalate. The two enzymes that are unique to the cycle, namely phosphoribulokinase and carboxydismutase, are synthesized during growth on formate but are not synthesized (except in two special instances) during growth on oxalate. This shows that a different pathway of carbon assimilation is operating during growth on these two substrates. The conclusion is borne out by the finding that [^{14}C]formate and [^{14}C]bicarbonate are incorporated only to a minor extent during growth on oxalate.

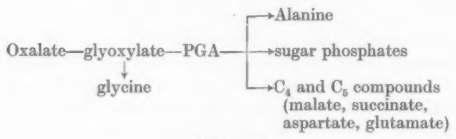
The possibility, nevertheless, remained that during the adaptation of the formate-grown organism to growth on oxalate the synthesis of enzyme(s) catalysing a decarboxylation of oxalate to formate and carbon dioxide, or a complete oxidation to carbon dioxide, might enable the formate-grown cell to use its existing enzymic apparatus temporarily to incorporate carbon dioxide by the ribulose 1:5-diphosphate cycle in an oxalate growth medium, before control mechanisms stopped the synthesis of key enzymes of this cycle in order to deflect the oxalate carbon into a more favourable assimilation pathway. The synthesis of carboxydismutase observed during the first generation of growth in oxalate by cells previously grown in formate suggested that this might be occurring. However, cells harvested at this particular stage fixed only small quantities of [^{14}C]bicarbonate, compared with the formate-grown organisms (J. R. Quayle & P. J. Large, unpublished observation), showing that despite the availability both of carbon dioxide and the enzymes necessary to incorporate it by the ribulose 1:5-diphosphate cycle, the operation of the cycle is inhibited.

The factors regulating carboxydismutase synthesis in cells growing on oxalate, subsequent to growth on formate, are not at present understood and need further investigation. The enzyme is synthesized by the cells during the first generation of growth on oxalate subsequent to their adaptation from formate. It seems unlikely that formate can be present in the growth medium after the lag period of 10 hr. that is entailed in the adaptation from growth on formate to growth on oxalate. Both formate- and oxalate-grown cells possess a formic dehydrogenase which would presumably enable them to oxidize the residual formate to carbon dioxide. In succeeding generations the enzyme is not longer synthesized but is 'diluted out'. These cells after seven generations of growth in the oxalate medium appear to possess some property different from cells which have been growing for a long time on oxalate. This is manifested by a rapid synthesis of carboxydismutase in response to addition of formate to the oxalate growth medium in the cells de-adapted from growth on formate, but not in the oxalate-grown cells. It is not known through how many bacterial generations of growth on oxalate, subsequent to growth on formate, this effect persists. Similar effects may have been observed recently by McCarthy (1959) during a study of the adaptation of *Aerobacter aerogenes* to growth on glucose or succinate. During the adaptation of this organism from growth on glucose to growth on succinate, the succinic-dehydrogenase activity rose approximately 20-fold after a lag period of 14 hr. Transfer of succinate-

grown cells to a glucose medium caused the succinic-dehydrogenase activity to fall to its original level, but not so rapidly as simple dilution of the cell contents with the normal material of glucose-grown cells would require, suggesting that the succinic-dehydrogenase system continued to be formed in excess of normal requirements for some time. When the cells were transferred to succinate medium after three generations of growth in the glucose medium they showed rapid synthesis of succinic dehydrogenase with no lag period, in contrast with cells grown a long time on glucose.

Apart from the special case discussed above, formate appears to act as an inducer of carboxydismutase synthesis only when cells are resuspended in a formate growth medium. If formate is added to a growth medium containing a carbon substrate other than formate, in which the organism is already growing, no detectable carboxydismutase synthesis takes place. No evidence was found for increased partial pressure of carbon dioxide raising the activity of carboxydismutase in *P. oxalaticus* or ribose-grown *E. coli*. The latter finding contrasts with the report of Fuller (1956) and Fuller & Gibbs (1959) of such an effect in xylose- or arabinose-grown *E. coli*.

The results of kinetic analysis of the entry of [^{14}C]oxalate into *P. oxalaticus* growing on oxalate suggest that carbon atoms are incorporated by a pathway which is characterized by the chain of metabolites shown in Scheme 1 (volatile intermediates would not have been detected):



Scheme 1

Studies with cell-free extracts indicate that the transformation of oxalate into PGA may occur by reduction of an 'activated' oxalate to glyoxylate, condensation and decarboxylation of 2 molecules of glyoxylate to 1 molecule of a C_3 compound which is then reduced and phosphorylated to PGA. This series of reactions would then place the glycine in Scheme 1 as arising from glyoxylate; the latter compound, whose presence would not be detected satisfactorily in the chromatographic and radioautographic analyses, actually being on the main pathway and not glycine.

Such a sequence of reactions has many features in common with that proposed for the growth of *Pseudomonas* species on glycollate (Kornberg & Gotto, 1959) and glycine (Callely & Dagley, 1959) respectively. The essential difference lies in the reduction of oxalate to glyoxylate, the latter compound being the first common intermediary metabolite of these three growth substrates.

The mechanism of the suggested oxalate activation is currently under investigation. The work with a cell-free extract implicates oxalyl-CoA by a transphorase reaction with acetyl-CoA. However, this cannot be the system used for oxalate activation by the whole cell as little acetate activation in the presence of ATP and CoA can be observed in cell-free extracts. Similarly, no direct oxalate activation in the presence of these cofactors has been observed. Recent work indicates that the oxalate is activated instead through a transphorase reaction with succinyl-CoA.

SUMMARY

1. The incorporation of carbon atoms from [^{14}C]oxalate, [^{14}C]formate or [^{14}C]bicarbonate into the aqueous ethanol-soluble fraction of *Pseudomonas oxalaticus* (OX 1) grown on oxalate has been studied.

2. Only isotope from [^{14}C]oxalate appeared in appreciable quantities in the aqueous ethanol-soluble fraction of the cells during 10 min. incubation periods. This shows that carbon dioxide-fixation processes, similar to those found to occur during growth of the organism on formate, do not play a major part during growth on oxalate.

3. This conclusion is supported by the findings that carboxydismutase and phosphoribulokinase are synthesized during growth on formate but not (except under special conditions) during growth on oxalate.

4. Carboxydismutase synthesis has been observed in cells growing on oxalate only if they have previously been growing on formate. Such cells continue to synthesize carboxydismutase during the first generation of growth on oxalate subsequent to their adaptation from growth on formate. Addition of formate to the oxalate growth medium leads to resumed enzyme synthesis. No enzyme is formed if the cells have not been grown previously on formate.

5. The radioactivity incorporated at the earliest times (3 sec.) from [^{14}C]oxalate was present mainly in glycine (65%) and 3-phosphoglyceric acid (25%). After incubation for 45 sec., radioactivity also appeared in alanine, glutamate, malate and phosphates of glucose, fructose and sedoheptulose.

6. The data show that the isotope enters glycine much more rapidly than it enters phosphoglycerate.

7. Cell-free extracts catalyse the formation of glyceric acid and carbon dioxide by a diphosphopyridine nucleotide-linked reductive condensation

of glyoxylate, or oxalate, in the presence of acetyl-coenzyme A.

8. It is suggested that during growth on oxalate, the oxalate, possibly in the form of oxalyl-coenzyme A, is reduced to glyoxylate, which is taken to be the precursor of glycine and phosphoglycerate. The phosphoglycerate formed then enters established routes of biosynthesis.

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The Interconversion of Glutamic Acid and Aspartic Acid in Respiring Tissues

BY H. A. KREBS AND D. BELLAMY

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

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In previous papers (Krebs, 1950; Terner, Eggleston & Krebs, 1950) it was briefly reported that glutamic acid, when added in excess to respiring animal-tissue preparations, is rapidly converted into aspartic acid, and that aspartic acid, when added in excess, is converted into glutamic acid. Qualitatively these interconversions are not unexpected: both amino acids are oxidized in animal tissues through the tricarboxylic acid cycle and therefore form α-oxoglutarate and oxaloacetate as intermediates. As animal tissues all contain aspartic-glutamic transaminase, any excess of the added amino acids may be expected to transaminate with the appropriate α-oxo acid arising as an intermediate. Starting with an excess of glutamate, a trace quantity of oxaloacetate present in the tissue, or formed from glutamate, would thus be expected to react as follows:

$$\begin{array}{l} \text{oxaloacetate} + \text{glutamate} \rightarrow \text{aspartate} + \alpha\text{-oxoglutarate} \\ \alpha\text{-oxoglutarate} + 1\tfrac{1}{2}\text{O}_2 \rightarrow \text{oxaloacetate} + \text{CO}_2 \\ \hline \text{Sum: glutamate} + 1\tfrac{1}{2}\text{O}_2 \rightarrow \text{aspartate} + \text{CO}_2 \end{array} \quad (1)$$

Analogously, starting with an excess of aspartate, a trace quantity of α-oxoglutarate and an endogenous substrate such as lactate, the expected sequence is:

$$\begin{array}{l}\alpha\text{-oxoglutarate} + \text{aspartate} \rightarrow \text{glutamate} + \text{oxaloacetate} \\ \text{oxaloacetate} + \text{lactate} + 1\tfrac{1}{2}O_2 \rightarrow \alpha\text{-oxoglutarate} + 2CO_2 \\ \hline \text{Sum: aspartate} + \text{lactate} + 1\tfrac{1}{2}O_2 \rightarrow \text{glutamate} + 2CO_2\end{array} . \quad (2)$$

In this case a source of acetyl-coenzyme A, such as lactate or a triose phosphate equivalent, a C_2-fragment of a fatty acid or second molecule of aspartate is required.

Whilst, then, qualitatively the interconversion of glutamate and aspartate is expected, the extent to which this occurs cannot be predicted because several other pathways, especially complete oxidation, are open to both α-oxoglutarate and oxaloacetate, and no information is available on the rate at which transamination occurs at the low concentrations of the α-oxodicarboxylic acids in animal tissues.

The data presented in this paper indicate that, under some conditions, transamination rather than complete oxidation of the α-oxo acids is the preferred route. Although the steady-state concentration of the α-oxo acids is very low, transamination occurs very readily.

METHODS

Manometric determination of aspartic acid. The manometric method of Crawford (1957) employing the specific aspartic decarboxylase of *Nocardia globerula* (National Collection of Industrial Bacteria, Teddington, Middlesex, no. 8852) was used. This enzyme converts L-aspartate into α-alanine. The aspartic decarboxylase of this organism was found to be much more active (by a factor of about 10) in washed suspensions than in the acetone-dried powder recommended by Crawford. On the other hand, washed suspensions, in contrast with acetone-dried powders, absorb O_2 under test conditions when added to tissue extracts. Although the rate of consumption of O_2 is very low, the error introduced by it can be significant but can be avoided by anaerobic conditions. The specificity of acetone-dried powder and washed suspensions appears to be equal, as identical aspartate values were obtained with both preparations in an analysis of casein hydrolysate. Asparagine did not react.

The yield of the organism was greatly increased when growth conditions were aerobic. The organism was therefore grown in Roux bottles containing 2 g. of peptone, 0·25 g. of Difco-yeast extract, 1 g. of glucose, 3 g. of agar and 100 ml. of water. The flasks were inoculated with a (5 ml.) liquid subculture, containing 0·5% of yeast extract, 0·9% of NaCl, 0·2% of sodium citrate and 0·1% of glucose. After 18 hr. at 30°, about 4·5 g. of wet cells was obtained from one Roux bottle. The cells were washed three times on the centrifuge with 0·9% NaCl and finally suspended in 4 vol. of 0·9% NaCl. Of this suspension, 0·5 ml. liberated the theoretical amount of CO_2 from 20 μmoles of aspartic acid in 20–40 min. Q_{CO_2} during the initial stages was about 180. The stock suspension was stored at −15°. Repeated thawing and freezing did not reduce the activity of the decarboxylase.

Replacing glucose by hydrolysed casein did not affect the yield of cells or the activity of the enzyme. Addition of asparagine to the growth medium reduced the decarboxylase activity, especially in the early stages of incubation.

For the determination of aspartate, up to 3 ml. of the solution to be tested was measured into the main compartment of a conical Warburg vessel, together with 0·3 ml. of 3M-acetate buffer, pH 5·0. The side arm contained 0·5 ml. of the *Nocardia* suspension and 0·1 ml. of the acetate buffer; the centre well contained phosphorus and the gas space N_2. The bath temperature was 37°.

Manometric determination of glutamic acid. The decarboxylase method with the glutamic decarboxylase of *Clostridium welchii*, strain S.R. 12, was used (see Krebs, 1950). The values obtained by this method are too high when both aspartic acid and α-oxo acids are present (see Müller & Leuthardt, 1950; Meister, Sober & Tice, 1951). Very low concentrations of pyruvate or α-oxoglutarate suffice to cause a decarboxylation of aspartic acid to α-alanine. This difficulty was overcome by removing aspartic acid first with *Nocardia* suspensions and carrying out the glutamate determination on the material in which aspartate had been determined.

Chromatographic determination of amino acids. The methods of Clayton & Strong (1954) and of Meyer (1957) were used. After incubation, the tissue suspension was added to an equal volume of boiling ethanol. The mixture was heated at 100° for a further 5 min. and then cooled and centrifuged at 25 000 *g* for 30 min. Samples of the supernatant fluid and standard solutions of amino acids were applied 8 cm. from one end of strips of Whatman no. 1 (chromatography grade) paper about 30 cm. long. They were placed 4 cm. apart and contained from 0·5 to 2·0 μg. of amino nitrogen. The chromatograms were developed with a descending solvent (Clayton & Strong, 1954) for about 8 hr.

Ninhydrin solution (Meyer, 1957) was prepared weekly by mixing a freshly prepared solution of 0·3 g. of ninhydrin in 100 ml. of 95% ethanol with a solution of 0·02 g. of ethylenediaminetetra-acetic acid in 6·5 ml. of water. After the papers were dried for 1 hr. in a stream of air at room temperature, the ninhydrin solution was sprayed lightly on to the paper. The chromatogram was heated at 110° until the coloured spots could just be detected (about 3 min.).

Each spot was cut out, divided into small pieces and placed in 10 ml. stoppered tubes with 3 ml. of ninhydrin reagent. The tubes were heated at 50° with occasional shaking for 45 min. The extinction of the resultant solution was measured at 578 mμ.

The chromatographic method was used in all experiments on mitochondria. In the experiment recorded in Table 2 manometric methods were also used.

Determination of α-oxo acids. α-Oxoglutarate was estimated as the 2:4-dinitrophenylhydrazone by the method of El Hawary & Thompson (1953), and oxaloacetate according to Kalnitsky & Tapley (1958).

Preparation and incubation of tissue homogenates. Tissue from a freshly killed animal was cooled in ice and homogenized with 4 vol. of 0·155M-KCl in a stainless-steel apparatus (type Potter & Elvehjem, 1936). For the incubation, 4 ml. of homogenate was measured into the main compartment of a large (50 ml.) conical Warburg vessel, together with 0·8 ml. of 0·1M-potassium phosphate buffer, pH 7·4, 0·8 ml. of 0·02M-$MgCl_2$, 0·8 ml. of 0·02M-adenosine triphosphate (ATP), varying quantities of 0·2, 0·4 or 0·75M-sodium glutamate and sufficient 0·155M-KCl to bring the volume to 8 ml. The centre well contained 0·3 ml. of 2N-NaOH and the gas space O_2. The vessels were shaken in a Warburg bath at 37° and the O_2 consumption was measured manometrically. At the end of the incubation period (1 hr.), the enzymic reaction was stopped by the addition of 2 ml. of 2N-HCl.

Micro-organisms. Escherichia coli, N.C.I.B. no. 8571, was grown at 30° for 24 hr. in a medium containing 1% of Lab-Lemco, 0·5% of NaCl, 0·5% of Difco-yeast extract, 2% of Tryptone, 2% of glucose and 3% of agar. *Alcaligenes faecalis* was grown as described by Krebs, Whittam & Hems (1957). The cells were washed twice on the centrifuge with water and finally resuspended in approx. 6 vol. of water. For measurement of the metabolism of glutamate, 2 ml. of washed cells (approx. 18 mg. dry wt.) was measured into the main compartment of a conical Warburg vessel together with 1 ml. of 0·1M-potassium phosphate buffer, pH 7·4, and glutamate to a final concentration of 0·02M. The volume was made up to 4·5 ml. with water.

Preparation of mitochondria. Rat- and pigeon-liver mitochondria were prepared according to the method of Werkheiser & Bartley (1957), except that the dissolved CO_2 was not removed from the sucrose solution. Rat-brain mitochondria were prepared by the method of Bellamy (1959) and pigeon-breast-muscle mitochondria according to the method of Chappell & Perry (1953). The mitochondria were finally suspended in 0·25M-sucrose. Each cup contained 2 ml. of mitochondrial suspension (about 400 mg. wet wt.); 0·4 ml. of 0·1M-potassium phosphate buffer, pH 7·4; 0·4 ml. of 0·02M-$MgCl_2$; 0·4 ml. of 0·02M-ATP, varying quantities of 0·4M-sodium glutamate and sufficient 0·25M-sucrose solution to bring the volume to 4·5 ml. The centre well contained 0·2 ml. of 2N-NaOH, the gas space air. The bath temperature was 25°.

RESULTS

Formation of aspartate from glutamate. Glutamate was added to homogenates of liver, breast muscle and kidney cortex and the oxygen consumption and the changes in the concentrations of glutamate and aspartate were measured (Tables 1–4). The addition of glutamate increased the

Table 1. *Effect of glutamate concentration on the conversion of glutamate into aspartate in rat-liver homogenate*

For experimental conditions see text. Results refer to μmoles in 8 ml. of suspension. Before addition of glutamate 8 ml. contained 8·8 μmoles of glutamate, 4·8 μmoles of aspartate and 4·6 μmoles of α-oxoglutarate. Glutamic acid and aspartic acid were determined manometrically.

	μmoles				
Glutamate added	—	152	304	456	608
Glutamate found	9·9	94	237	349	519
Aspartate found	2·7	64·0	77·5	72·4	64·0
α-Oxoglutarate found	2·3	9·1	7·7	20·4	16·0
Changes:					
O_2	−71	−200	−192	−183	−156
Glutamate	+ 1·1	−67	−76	−116	− 98
Aspartate	− 2·1	+ 59·2	+ 72·7	+ 67·6	+ 59·2
α-Oxoglutarate	− 2·3	+ 4·5	+ 3·1	+ 15·8	+ 11·4

Table 2. *Effect of glutamate concentration on the conversion of glutamate into aspartate in homogenate of pigeon-breast muscle*

For experimental conditions see text. Results refer to μmoles in 8 ml. of suspension. Before addition of glutamate 8 ml. contained 5·1 μmoles of glutamate and 2·2 μmoles of aspartate. Amino acids were estimated manometrically (m) and chromatographically (c). The calculation of the changes is based on the manometric determinations.

	μmoles								
Glutamate added	—	152		304		456		608	
	(m)	(m)	(c)	(m)	(c)	(m)	(c)	(m)	(c)
Glutamate found	1·4	60·8	53·9	212	194	386	342	538	536
Aspartate found	0·6	70·4	72·7	98·2	83·7	60·8	56·5	49·0	47·5
Changes:									
O_2	−95	−189		−161		−139		−150	
Glutamate	− 3·7	−96		− 97		− 75		− 75	
Aspartate	− 1·6	+ 68·2		+ 96·0		+ 58·6		+ 46·8	

Table 3. *Effect of glutamate concentration on the conversion of glutamate into aspartate in homogenate of pigeon liver*

For experimental conditions see text. Results refer to μmoles in 8 ml. of suspension. Before addition of glutamate 8 ml. contained 12·9 μmoles of glutamate and 5·6 μmoles of aspartate. Glutamic acid and aspartic acid were determined manometrically.

	μmoles				
Glutamate added	—	152	304	456	608
Glutamate found	7·5	78	194	418	574
Aspartate found	5·6	65·0	95·5	31·2	19·6
Changes:					
O_2	−90	−224	−184	−129	−136
Glutamate	− 5·4	− 87	−123	− 51	− 47
Aspartate	0	+ 59·4	+ 89·9	+ 25·6	+ 14·0

Table 4. *Effect of glutamate concentration on the conversion of glutamate into aspartate in homogenate of sheep-kidney cortex*

For experimental conditions see text. Results refer to μmoles in 8 ml. of suspension. Before addition of glutamate, 8 ml. contained 3·6 μmoles of glutamate and less than 0·2 μmole of aspartate. Glutamic acid and aspartic acid were determined manometrically.

	μmoles				
Glutamate added	—	76·5	153	316	632
Glutamate found	0·2	34·1	101·5	263	562
Aspartate found	6·2	42·6	55·3	61·6	70·1
Changes:					
O_2	−61·4	−145	−168	−195	−215
Glutamate	− 3·4	− 46	− 55	− 57	− 74
Aspartate	+ 6·2	+ 42·6	+ 55·3	+ 61·6	+ 70·1

oxygen consumption in all four tissues, the increases being greater with 150 and 300 μmoles of glutamate than with 450 and 600 μmoles, except in kidney cortex, where oxygen consumption continued to increase with the glutamate concentration. Much of the added glutamate was removed during the incubation and much aspartate was formed. Under several conditions, the glutamate oxidized was almost quantitatively recovered as aspartate, e.g. with rat liver and pigeon-breast muscle with 300 μmoles of glutamate and at all glutamate concentrations in kidney cortex. The total (uncorrected for endogenous) oxygen consumption was generally greater than expected from reaction (1) but in some experiments, e.g. when about 300 μmoles of glutamate was added to rat liver or muscle, the excess of oxygen used was small. This indicates that the main oxidative reaction which occurred was the oxidation of glutamate to aspartate according to reaction (1).

The level of α-oxoglutarate was measured at the end of the incubation in the experiment shown in Table 1. It increased in the presence of glutamate and was of the order of 1–2 mM.

The level of oxaloacetate was measured in another experiment and was below the capacity of the method, i.e. below 0·05 mM. The transamination between glutamate and oxaloacetate thus readily occurred at very low oxaloacetate concentrations.

Formation of glutamate from aspartate. Like glutamate, aspartate increased the rate of oxygen consumption of the four tissues (Tables 5–8). The amounts of aspartate removed generally rose with the aspartate concentration except in sheep-kidney cortex, where the rate of aspartate removal was not much affected by the concentration of aspartate. The maximum rates of aspartate removal were lower than those for glutamate, about half in rat liver, pigeon-breast muscle and sheep-kidney cortex and a quarter in pigeon liver. The amounts of glutamate formed on addition of aspartate were between 40 and 60 % of the aspartate removed. A value of 50 % would be expected if both the oxaloacetate and the acetyl-coenzyme A needed for the synthesis of glutamate were derived from aspartate. The total amounts of oxygen used were rather greater than those required according to reaction (2), save in rat liver at high aspartate concentrations. In this tissue, most of the total oxygen consumption was due to the oxidation of aspartate to glutamate; in kidney roughly 50 % accounted for this reaction. The α-oxoglutarate level at which ready transamination took place was measured in the kidney experiments and found to be about 2 μmoles in 8 ml., or 0·25 mM.

Conversion of glutamate into aspartate in mitochondria. As shown in Tables 9 and 10, a conversion of glutamate into aspartate also occurred in isolated mitochondria but the yield of aspartate was

Table 5. *Effect of aspartate concentration on the conversion of aspartate into glutamate in homogenate of rat liver*

For experimental conditions see text. Results refer to μmoles in 8 ml. of suspension. Before addition of aspartate 8 ml. contained 5·7 μmoles of glutamate and 5·3 μmoles of aspartate. Glutamic acid and aspartic acid were determined manometrically.

	μmoles				
Aspartate added	—	160	320	480	640
Aspartate found	4·1	150	294	425	590
Glutamate found	2·7	12·3	23·8	41·6	32·5
Changes:					
O_2	−39·0	−67·0	−79·6	−80·2	−78·0
Aspartate	− 1·2	−15	−31	−60	−55
Glutamate	− 3·0	+ 6·6	+18·1	+35·9	+26·8

Table 6. *Effect of aspartate concentration on the conversion of aspartate into glutamate in homogenate of pigeon-breast muscle*

For experimental conditions see text. Results refer to μmoles in 8 ml. of suspension. Before addition of aspartate 8 ml. contained 5·5 μmoles of glutamate and 1·7 μmoles of aspartate. Glutamic acid and aspartic acid were determined manometrically.

	μmoles				
Aspartate added	—	160	320	480	640
Aspartate found	2·6	138	285	429	596
Glutamate found	0·4	15·7	31·6	38·8	30·3
Changes:					
O_2	−97	−146	−153	−147	−139
Aspartate	+ 0·9	− 24	− 37	− 53	− 46
Glutamate	− 5·1	+ 10·2	+ 26·1	+ 33·3	+ 24·8

Table 7. *Effect of aspartate concentration on the conversion of aspartate into glutamate in homogenate of pigeon liver*

For experimental conditions see text. Results refer to μmoles in 8 ml. of suspension. Before addition of aspartate 8 ml. contained 5·7 μmoles of glutamate and 3·1 μmoles of aspartate. Glutamic acid and aspartic acid were determined manometrically.

	μmoles				
Aspartate added	—	160	320	480	640
Aspartate found	2·4	155	310	469	615
Glutamate found	1·3	17·8	13·2	14·2	21·6
Changes:					
O_2	−55	−69	−76	−87	−109
Aspartate	−0·7	− 8	−13	−14	− 28
Glutamate	−4·4	+12·1	+ 7·5	+ 8·5	+ 15·9

Table 8. *Effect of aspartate concentration on the conversion of aspartate into glutamate in homogenate of sheep-kidney cortex*

For experimental conditions see text. Results refer to μmoles in 4 ml. of suspension. Before addition of aspartate, 4 ml. contained 3·1 μmoles of glutamate, 1·5 μmoles of aspartate and 0·2 μmole of α-oxoglutarate. Glutamic acid and aspartic acid were determined manometrically.

	μmoles					
Aspartate added	—	75	150	300	450	600
Aspartate found	1·0	47·0	126	273	424	576
Glutamate found	0·8	19·9	20·4	21·0	19·0	16·0
α-Oxoglutarate found	0·2	1·3	0·9	1·2	1·0	1·0
Changes:						
O_2	−34·2	−54·0	−62·0	−62·0	−66·0	−69·0
Aspartate	− 0·5	−29·5	−25·5	−28·5	−27·5	−25·5
Glutamate	− 2·3	+16·8	+17·3	+17·9	+15·9	+12·9
α-Oxoglutarate	0	+ 1·1	+ 0·7	+ 1·0	+ 0·8	+ 0·8

very low with mitochondria prepared from rat liver, pigeon liver and pigeon-breast muscle. In contrast, with rat-brain mitochondria most of the glutamate removed was recovered as aspartate, the yield reaching 91% when 80 μmoles of glutamate was added to 4·5 ml. of suspension (Table 9). Under these conditions, the changes observed are fairly close to those expected according to reaction (1). This finding indicates that the brain mitochondria possessed a high transaminase activity. The low yield of aspartate in liver and muscle mitochondria was presumably due to loss of transaminase in the washing process.

Müller & Leuthardt (1950), using paper chromatography, have already demonstrated qualitatively a conversion of glutamic acid into aspartic acid in rat-liver mitochondria.

Oxidation of glutamate by micro-organisms. As many bacteria can oxidize glutamic acid by the same route as animal tissue and possess transaminase activity, experiments were carried out to test whether a ready conversion of glutamate into aspartate occurs in bacteria.

E. coli and *A. faecalis* both readily oxidized added glutamate, as shown by an increased oxygen consumption and removal of glutamate, but no accumulation of aspartate occurred. The data on the oxygen consumption of glutamate removal indicated that most of the glutamate which was removed underwent complete oxidation. Thus with the *Alcaligenes* strain, the suspension consumed 43·4 μmoles of oxygen with a removal of 8·8 μmoles of glutamate and a formation of less than 0·4 μmole of aspartate.

It should be mentioned that Bovarnick & Miller (1950) found a conversion of glutamic acid into aspartic acid in typhus rickettsiae.

DISCUSSION

The experiments show that the four tissues tested readily interconvert glutamate and aspartate. The observations are in agreement with the assumption that glutamate yields oxaloacetate, and aspartate yields α-oxoglutarate, through the reactions of the tricarboxylic acid cycle, and that the α-oxo acids form the respective amino acids by transamination with the starting material [see also Müller & Leuthardt (1950) and Klingmüller (1955)]. The steady-state levels of α-oxoglutarate were of the order of 1 mM and those of oxaloacetate below 0·1 mM. Under many conditions, transamination was the metabolic reaction preferred to those of the tricarboxylic acid cycle.

Animal tissues contain relatively large quantities of free glutamic acid (2–10 μmoles/g.; Krebs, Eggleston & Hems, 1949) and relatively small amounts of aspartate (0·1–0·4 μmole/g.; Krebs, 1950). The ready formation of aspartate from glutamate raises the question of the function of

Table 9. *Conversion of glutamate into aspartate in mitochondria*

For experimental conditions see text. Results refer to μmoles in 4·5 ml. of suspension. Before addition of glutamate, the mitochondrial suspension contained the following amounts of glutamate and aspartate: rat liver, 1·0, 0·4 μmole; rat brain, 3·1, 2·4 μmoles; pigeon liver, 1·7, 0·7 μmole; pigeon-breast muscle, 0·4, 0·4 μmole.

	Rat liver		Rat brain		Pigeon liver		Pigeon-breast muscle	
Glutamate added	—	80	—	80	—	80	—	80
Glutamate found	4·6	56·5	0·7	43·4	2·1	65·0	0·8	70·8
Aspartate found	2·1	4·8	1·1	38·6	2·6	5·9	0·1	2·4
Changes:								
O_2	−27·1	−61·4	−14·7	−60·2	−19·0	−40·3	−7·9	−25·0
Glutamate	+ 3·6	−24·5	− 2·4	−39·7	+ 0·4	−16·7	+0·4	− 9·6
Aspartate	+ 1·7	+ 4·4	− 1·3	+36·2	+ 1·9	+ 5·2	−0·3	+ 2·0

Table 10. *Effect of glutamate concentration on the conversion of glutamate into aspartate in rat-brain mitochondria*

For experimental conditions see text. Results refer to μmoles in 4·5 ml. of suspension. Before addition of glutamate, the mitochondrial suspension contained 2·7 μmoles of glutamate and 1·3 μmoles of aspartate.

	μmoles			
Glutamate added	—	80	160	240
Glutamate found	1·0	46·6	105	178
Aspartate found	0·5	34·7	44·1	52·2
Changes:				
O_2	−18·3	−69·2	−86·0	−85·2
Glutamate	− 1·7	−36·1	−57·7	−64·7
Aspartate	− 0·8	+33·4	+42·8	+50·9

aspartate formed from glutamate. It is relevant in this context that the amino group of several compounds synthesized in the tissues is known to be derived from aspartate. Examples are: the formation of arginine from citrulline, a step in the synthesis of urea in the mammalian liver (Ratner, Petrack & Rochovansky, 1953; Ratner, Anslow & Petrack, 1953; Ratner & Petrack, 1956) and in the synthesis of guanidinoacetic acid in kidney cortex (Borsook & Dubnoff, 1941; Krebs, 1942); the amidation of 5-amino-4-carboxyiminazole ribotide to yield 5-amino-4-carboxy-amidoiminazole ribotide, one of the steps of the synthesis of uric acid in birds (Lukens & Buchanan, 1957; Miller, Lukens & Buchanan, 1957); the amination of inosinic acid, leading to adenylic acid (Carter & Cohen, 1955; Abrams & Bentley, 1955; Lieberman, 1956). The reactions investigated in this paper can account for the supply of the aspartate required for these syntheses.

Cravioto, Massieu & Izquierdo (1951) have shown that in insulin shock the glutamate concentration of the brain falls and the aspartate concentration rises. The changes of the aspartate and glutamate concentration were of the same order of magnitude and of opposite sign, and this indicates that in insulin shock the same reactions occur *in vivo* as are described in this paper as occurring in isolated brain mitochondria.

SUMMARY

1. Homogenates of rat and pigeon liver, pigeon-breast muscle and sheep-kidney cortex convert added glutamate into aspartate. Under some conditions, the amounts of glutamate removed and aspartate formed are approximately equal. The homogenates also convert aspartate into glutamate, the amounts of glutamate formed being of the order of 50% of the amounts of aspartate removed. In many cases, reactions (1) and (2) account for most of the oxygen consumption in the presence of glutamate or aspartate.

2. An almost stoicheiometric conversion of glutamate into aspartate also occurs in rat-brain mitochondria. In mitochondria of rat and pigeon liver and of pigeon-breast muscle, the yield of aspartate was only 20–30% of the glutamate removed.

3. Washed suspension of *Escherichia coli* and *Alcaligenes faecalis* did not accumulate aspartate on addition of glutamate.

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Studies on the Proteins of Fish Skeletal Muscle

7. DENATURATION AND AGGREGATION OF COD MYOSIN*

By J. J. CONNELL

Torry Research Station, Aberdeen, Department of Scientific and Industrial Research

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Earlier papers in this series (Connell, 1958*a*, *b*) showed that, compared with rabbit myosin, in the ease with which it aggregates cod myosin appears to be markedly unstable. Since myosin is the principal myofibrillar protein it seems likely that this instability is related to the general experience that the structural organization of cod muscle is much more easily damaged by, for example, dehydration or freezing than is mammalian muscle. Freezing and storing myosin or actomyosin in the frozen state produce complex aggregation and disaggregation reactions (Nord, 1936; Snow, 1950; Connell, 1959) that are little understood.

It was therefore decided to examine in detail the processes of denaturation and aggregation in solutions of myosin and to study the effects on these processes of varying conditions such as protein concentration, ionic strength and pH which are likely to alter markedly during freezing and drying.

An attempt has been made to measure both the intramolecular changes and the intermolecular interactions which occur during the storage of unfrozen solutions of cod myosin at a temperature, in the main, of 0°. While this work was nearing its completion Lowey & Holtzer (1959) published the results of an investigation of the aggregation of rabbit myosin which, though differing in scope from the present work, complements it in some particulars.

EXPERIMENTAL

Preparation of cod myosin

Some of this work has been carried out on cod myosin prepared by dissociating cod actomyosin and separating the liberated myosin by means of preparative ultracentrifuging (Connell, 1958*b*). The maximum concentration of pure myosin which can be prepared by this method is about 0·5%, though concentrations up to 0·8% can be prepared at the sacrifice of some contamination with actomyosin. As it was desirable to investigate more concentrated solutions than this, another method of preparing cod myosin has been mostly used. In this, the coarsely minced skeletal muscle of cod (*Gadus callarias* L.) is washed three or four times in the cold with phosphate buffer, pH 7·5, I 0·05 (Connell, 1958*b*) to remove the sarcoplasmic proteins, and the residue is then extracted at 0° by slowly stirring for 1 hr. with 3–4 vol. of 0·45 M-KCl–3·38 mM-KH_2PO_4–15·5 mM-Na_2HPO_4–10 mM-tetrasodium pyrophosphate–5 mM-$MgCl_2$, pH 7·5. Such extracts contain 1·5–1·8% of protein and generally show a single boundary in the ultracentrifuge (Fig. 1*a*) sedimenting with a velocity characteristic of myosin. Occasionally a much smaller and faster component is observed in the ultracentrifuge, presumably actomyosin or actin. Presumably such extracts also contain the 'extra-protein' fraction of muscle (Szent-Györgyi, Mazia & Szent-Györgyi, 1955). The amount of myosin in the extracts, judged by the area under the ultracentrifuge peak, is about 75–80% of the total protein content. The electrophoresis diagram of such a preparation showed a single, sharp main peak and a smaller (5–10% of the total area) faster peak. The values for some of the properties of the preparation, e.g. optical rotation, intrinsic viscosity, differ slightly from those of pure cod myosin (Connell, 1958*b*), providing an additional indication of the presence of impurities. However, no attempts were made to purify further this type of preparation since for the present investigation it was considered to be sufficiently pure. The usual dilution method of purifying myosin cannot be used with cod (Connell, 1958*a*, *b*); as will be shown below, fractionation with ammonium sulphate is impracticable because salting out renders the protein insoluble.

All the protein preparations have been made from muscle (sometimes frozen) which was excised before the onset of *rigor mortis*. The myosin concentrations referred to in this paper are calculated from the total protein nitrogen of the solution.

Methods

The methods used for measuring diffusion, sedimentation, optical rotation and viscosity have been described previously (Connell, 1958*b*), with the exception that in most of the sedimentation runs a phase plate was used in place of the diagonal wire or bar. All sedimentation runs were at between 0° and 5°, and viscosity and diffusion were measured at 0·5°.

The areas under the peaks of sedimentation pictures have been measured, after subtraction of appropriate base lines, either directly from the photographic plate with a two-way travelling microscope and trapezoidal integration, or, when comparison of areas in arbitrary units only was required, from photographic enlargements of the image followed by planimetry or by cutting out and weighing.

Adenosine triphosphatase activity was determined at 25° essentially by Perry's (1955) method. With the present myosin preparations the amount of hydrolysis of adenosine triphosphate (ATP; Sigma Chemical Co.) was linear with time up to 10 min. incubation and therefore usually only the amount of hydrolysis at 10 min. was measured.

* Part 6: Connell & Howgate (1959).

Digestion of myosin by trypsin (L. Light and Co. Ltd.) was followed in 0·45 M-KCl–3·38 mM-KH_2PO_4–15·5 mM-Na_2HPO_4, pH 7·5, by measuring the liberation of ninhydrin-reacting compounds, Yemm & Cocking's (1955) reagent being used after precipitation of protein with trichloroacetic acid.

Sulphydryl groups were determined at 0° by titration with mM-*N*-ethylmaleimide or mM-*p*-chloromercuribenzoate, the nitroprusside pink colour being used as indicator.

Ultraviolet-absorption spectra were measured in a Unicam SP. 500 spectrophotometer with matched 1 cm. cells.

RESULTS

General course of cod-myosin aggregation

The process of aggregation of rabbit myosin has been well described by Lowey & Holtzer (1959) and the stepwise side-by-side nature of the reaction demonstrated mainly by the light-scattering method. Fig. 1 shows the changes in the sedimentation picture which occur when cod myosin is stored at 0°; similar changes occur within the temperature range 0–23°. In dilute solutions of greatly aggregated myosin sometimes as many as five discrete peaks are visible. That the changes are a reflexion of a real increase in molecular weight (M) and not, for example, an unlikely but possible stepwise alteration of shape at constant M has been shown in the present case by measurements of the diffusion coefficient, D (Fig. 2) and of weight-average M (Fig. 3). The latter values were obtained by the Archibald procedure (Connell, 1958*b*). For aggregated samples, M falls progressively during the run owing to the removal of heavier components from the meniscus, and in order to find M of the uncentrifuged solutions the values obtained during any particular run were extrapolated to zero time. The decrease in D [as reflected in progressively smaller values of $(1/t).(1/h^2)$] coupled with the increase in S during storage can only mean that M is increasing, a conclusion substantiated by the results of the Archibald experiments. The form of the curve shown in Fig. 3 is different from that of the curves of increase of weight-average molecular weight as measured by light-scattering [Lowey & Holtzer's (1959), figs. 5, 6], which show that the rate of increase falls off gradually. This disparity might be accounted for by the very rapid removal of large aggregates from the meniscus in the Archibald experiments, which would tend to make the results lower than they should be, especially in the earlier stages of aggregation.

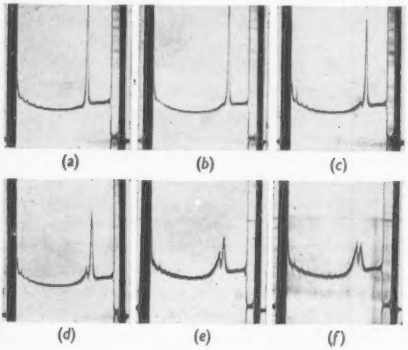

Fig. 1. Ultracentrifuge diagrams of a cod-myosin solution stored at 0°, pH 7·5, I 0·5 for (*a*) 0, (*b*) 1, (*c*) 2, (*d*) 3, (*e*) 6 and (*f*) 10 days. Sedimentation from right to left. (*a*) 87 min. and (*b*)–(*f*) 69 min. after reaching full speed (56 100 rev./min.). Temperature of runs, 3·3°. Protein concentration, 0·43 %.

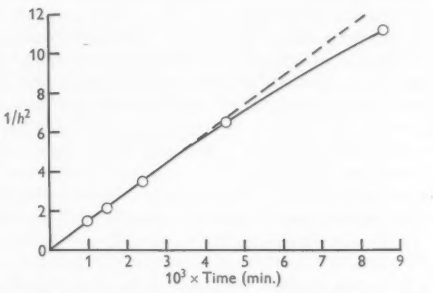

Fig. 2. Diffusion of a cod-myosin solution at 0·5°, pH 7·5, I 0·5. Protein concentration, 0·87 %. h, Maximum ordinate (in arbitrary units) of the diffusing peak.

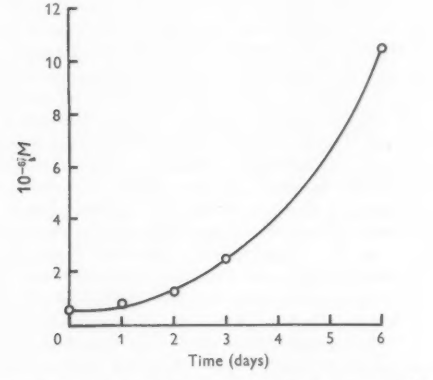

Fig. 3. Increase of weight-average molecular weight (M) during storage of cod myosin at 0°, pH 7·5, I 0·5, as measured by the Archibald procedure. Protein concentration, 0·79 %.

After the stage when the monomeric myosin peak has disappeared from the ultracentrifuge diagram, the aggregates continue to grow in size as indicated by a progressive increase in average S. The total area under the peaks of the ultracentrifuge diagram does not change much even when the monomer peak has disappeared, which suggests that the formation of 'precipitable' aggregates is slow. However, cod myosin does precipitate if kept long enough and it has been found, for example, that if a 1·5% solution of the protein is kept overnight at 23° and then for about a week at 0° it is converted into a rigid gel.

Kinetics of the aggregation

The detailed course of the aggregation has been followed by measuring the rate of disappearance of the monomeric myosin. The accuracy of this procedure is limited by a number of factors. The first is the shape of the myosin 'peak', which tends to be very sharp and difficult to define at the top. Secondly, owing to the sector-dilution effect, uncorrected areas at the photographic plate are not true measures of protein concentrations in the uncentrifuged solution. No attempt has been made to correct areas for this effect, which has been minimized by performing runs under as similar conditions of protein concentration, ionic strength, temperature, centrifugal field and time as possible, so that the peak is always measured at very nearly the same level in the cell. Areas of the monomer peak in aggregated solutions were then expressed as a fraction of the area of the monomer peak in the initial, unaggregated solution of myosin run under identical conditions. (In this respect Fig. 1*a* is exceptional in that the duration of the run is longer than the others in the series.) As the amount of monomer becomes less, under a given set of conditions its boundary will travel to a position slightly further down the cell (because of the inverse dependence of S for myosin on concentration), and the correction necessary for dilution will become larger. Neglect of this last effect is estimated to be at most 5% at the lowest concentrations measurable. Thirdly, the correction necessary because of the effect described by Johnston & Ogston (1946) will become greater as the ratio of monomer to aggregates becomes smaller (i.e. as the amount of monomer diminishes). Neglecting sector-dilution and assuming that the mixture contains only monomer and dimer, it was calculated from a typical set of results at a total protein concentration of 0·5% that the factor necessary to correct the observed to the actual concentration of monomer was 0·95, 0·89 and 0·67, when the amounts of monomer fell to 43, 28 and 15% respectively of their original amount. Above 50% of monomer remaining the factor is negligible whereas below this stage there will be a tendency for the Johnston & Ogston correction to be balanced by the dilution correction. However, the uncertainty involved in these corrections means that even corrected data below 20% of monomer remaining are likely to be considerably in

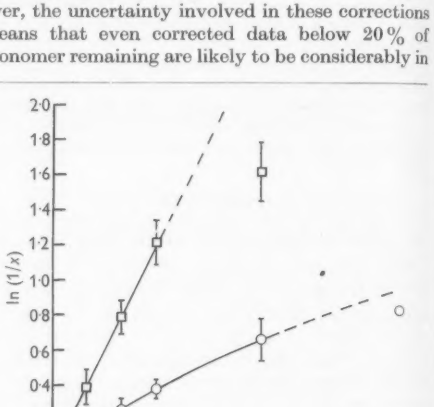

Fig. 4. First-order rate of disappearance of cod-myosin monomer during storage at 0°, pH 7·5, I 0·5. □, Mean values of four experiments run at protein concentrations of 1·2–1·5%; ○, mean values of six experiments run at protein concentrations of 0·3–0·5%. x, Fraction of the initial amount of monomer remaining. Vertical bars represent the standard deviations of the results; only two results were obtained at 10 days.

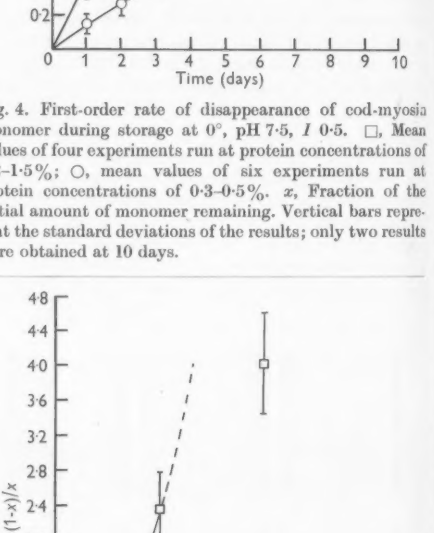

Fig. 5. Second-order rate of disappearance of cod-myosin monomer during storage at 0°, pH 7·5, I 0·5. Symbols are the same as in Fig. 4.

error, and, in fact, measurements have not been carried beyond this stage.

If the data from experiments such as that shown in Fig. 1 are analysed by means of the integrated forms of the first- or second-order-rate equations, the plots shown in Figs. 4 and 5 result. For more concentrated solutions (1·2–1·5 %) the data fit the first-order plot down to 30–40 % of monomer remaining; below this stage the order always becomes higher than unity and this is true even when the full Johnston & Ogston correction is applied. For dilute solutions (0·3–0·5 %) there is a tendency for the data during the initial stage of the reaction to fit the second-order plot slightly better than the first-order plot; at 40 % of monomer remaining the order is greater than unity.

For a given set of conditions the rate of disappearance of monomer varies erratically, though not very widely, from one preparation to another. A similar phenomenon was noted by Lowey & Holtzer (1959). This behaviour has necessitated making as many comparisons of different conditions as possible on the same preparation.

For any one preparation of myosin it has been found repeatedly that the first-order constant of disappearance is concentration-dependent over a wide range of concentrations, the rate increasing markedly at higher concentrations (Fig. 4). Extraneous factors that might influence the rate of aggregation in these experiments have been minimized by diluting the same preparation of myosin to different degrees, dialysing all the diluted solutions in the same buffer at 0° and then analysing them within a few hours of each other after adjusting the protein concentration to that of the most dilute solution.

This finding is in contrast with the observation of Lowey & Holtzer (1959) that the rate of aggregation of rabbit myosin as measured by light-scattering was (for two low concentrations of 0·02 and 0·04 %) independent of the initial concentration. It is possible that the reconciliation of these results lies in the fact that the absolute concentrations studied are so different. The present results suggest that the rate of aggregation is relatively constant at the lower concentrations examined and it is possible that at the very low concentrations studied by Lowey & Holtzer (1959) the rates as measured by light-scattering, which is particularly sensitive to large aggregates, would appear the same.

For the initial portion of the first-order-rate curve the reaction constants at 0° from the mean results of several experiments varied between $0{\cdot}14 \times 10^{-5}$ sec.$^{-1}$ for a 0·3–0·5 % solution and $0{\cdot}45 \times 10^{-5}$ sec.$^{-1}$ for a 1·2–1·5 % solution in 0·45 M-KCl–phosphate, pH 7·5, I 0·05. Although an exact comparison is not possible because the conditions are so different, Lowey & Holtzer (1959) calculate a first-order-rate constant of $1{\cdot}8 \times 10^{-5}$ sec.$^{-1}$ from the rate of increase of the average M determined by light-scattering during the aggregation of rabbit myosin at 0·02 and 0·04 % and at 25° (0·6 M-KCl, pH 6·3). At 4° it is stated (Lowey & Holtzer, 1959) that the rate is about one-tenth of that at 25°.

In order to compare reaction rates under different conditions, the protein concentration has been kept constant in any one experiment (usually at about 0·3–0·4 %) and the rates quoted are for convenience those calculated from the initial portions of the first-order-rate curve, i.e. before the stage when about 30 % of the monomer remains.

Effect of temperature, ionic strength and pH *on the aggregation*

Temperature. At 0°, 3·5° and 8°, one preparation (concentration 0·48 %) aggregated at first-order rates of 0·12, 0·21 and $0{\cdot}75 \times 10^{-5}$ sec.$^{-1}$ respectively, corresponding to an activation energy of approx. 41 kcal.mole^{-1}. Almost no monomer was observed in the ultracentrifuge diagram of a 1·5 % solution which had stood 18 hr. at 23°.

Ionic strength. The effect of ionic strength is shown in Fig. 6. In order to avoid high local concentrations of salt, portions of the stock myosin solution were diluted with an equal volume of phosphate buffer, pH 7·5, I 0·05, containing sufficient KCl to bring the final ionic strength to 0·3, 0·5, 1·0, 2·0 and 3·0, and then the diluted solutions were dialysed against KCl–phosphate buffer of total ionic strength 0·3, 0·5, 1·0, 2·0 and 3·0 respectively. With the solution diluted to I 0·3 this procedure resulted in the precipitation of about 10 % of the protein.

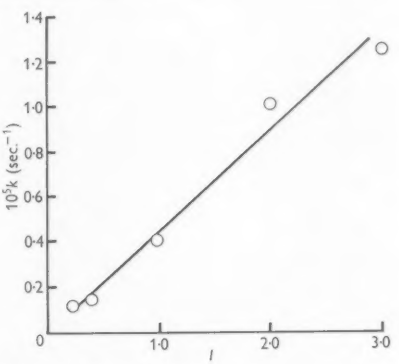

Fig. 6. Effect of ionic strength on aggregation of cod myosin at 0°, pH 7·5. Protein concentration, 0·47 %. k, First-order-rate constant calculated as described in the text.

The increase of rate of aggregation at higher ionic strength is in line with Lowey & Holtzer's (1959) observations that the rate is greater in 1·5 than 0·6M-KCl. Exposure of cod myosin to I 1·0 or above produces small amounts of a polydisperse ultracentrifugal component of low S. More of this material is produced at higher ionic strengths though the amount never exceeded about 10% of the total protein present. As described below, this component, or one like it, often appears when cod myosin is subjected to extreme conditions. It is possibly a dissociation product of low M like that observed by Kominz, Carroll, Smith & Mitchell, (1959) in alkali-treated rabbit myosin.

Precipitation of cod myosin once, by diluting with water, results in its irreversible aggregation (Connell, 1958*a*, *b*). The protein becomes progressively more difficult to dissolve when repeatedly precipitated in this way and that which does dissolve always shows considerable amounts of aggregated material in the ultracentrifuge. In addition, salting-out by half-saturation with ammonium sulphate renders cod myosin insoluble.

pH. As shown in Fig. 7, aggregation is minimal between pH 7·0 and 8·0 and increases on both sides of this range. Below pH 5·5–5·7, of course, the protein precipitates in the presence of salt. Below pH 6·2 and above pH 9·2 at I 0·5 progressively more of the polydisperse component of low S accompanies the increased tendency to aggregate.

Cod myosin which has been precipitated by dilution and then washed with water can be dissolved in acetate buffer (I 0·05, pH 4). In this solvent the protein (concentration 0·41%) shows a single asymmetric ultracentrifuge component of $S_{20,w} = 4{\cdot}1 \times 10^{-13}$ sec. Presumably under these conditions the protein is considerably denatured because it has $[\alpha]_D^{25} - 78°$ [compared with $[\alpha]_D^{23} - 29°$ and $-104°$ for native and urea-denatured cod myosin respectively at I 0·5, pH 7·5 (Connell, 1958*b*)], and on returning it to neutrality or adding salt it precipitates.

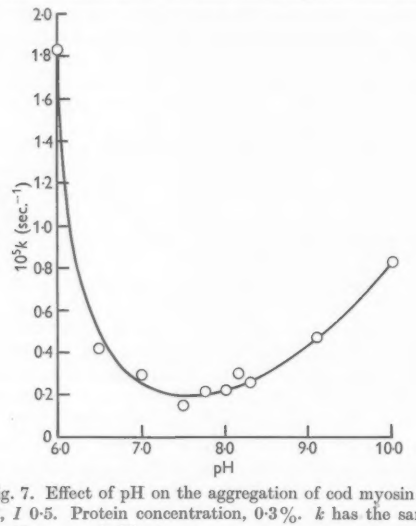

Fig. 7. Effect of pH on the aggregation of cod myosin at 0°, I 0·5. Protein concentration, 0·3%. k has the same significance as in Fig. 6.

Effects of additives on the aggregation

Sulphydryl reagents. Lowey & Holtzer (1959) reported that oxidation with sulphite or treatment with mM-thioglycollate did not affect the rate of aggregation of rabbit myosin. Connell (1959) noted that the aggregation of cod myosin is not inhibited by 10 mM-monothioglycol. As shown in Fig. 8, 0·1M-monothioglycol accelerates aggregation whereas 0·1M-ethylene glycol is without effect. It has also been found that oxidation by iodosobenzoic acid or alkylation by *N*-ethylmaleimide or *p*-chloromercuribenzoate increases the rate of aggregation of cod myosin. Aggregation does not therefore seem to occur by reactions involving the 'reactive' sulphydryl groups. Since the sulphydryl reagents described react with only about 50% of the total sulphydryl groups of cod myosin (J. J. Connell, unpublished work) it is conceivable that the aggregation may occur through a sulphydryl–disulphide-exchange reaction involving the remaining sulphydryl groups.

The accelerating effects of the sulphydryl reagents suggests that the sulphydryl and disulphide groups are necessary for the stability of the protein.

Urea and sodium dodecyl sulphate. In experiments with these reagents care was taken that the protein was not exposed to concentration greater than the final one aimed at.

Urea concentrations from 0·3 to 1·0M accelerate aggregation (Fig. 9). Polydisperse material of low S is evident even at 0·3M and the amount of this increases with the urea concentration. This fact, together with the pronounced curvature of the base line in ultracentrifuge runs at high urea concentrations, makes the interpretation of effects above 1·0M uncertain. However, as judged from the amounts of aggregates formed after equivalent periods of storage there are indications that aggregation is retarded or, at least, not accelerated, in 2M-urea and above (Fig. 9).

Sodium dodecyl sulphate at a concentration of 0·04% does not precipitate the protein but produces large amounts of an ultracentrifuge component of $S_{20,w}$ 14×10^{-13} sec., presumably an aggregate different in type from that produced during normal storage. Sodium dodecyl sulphate at this concentration also causes the formation of polydisperse material of low S.

Actin. Fig. 10 demonstrates that keeping myosin in the form of actomyosin has a considerable protective effect.

Changes in viscosity, optical rotation, sulphydryl groups and ultraviolet-absorption spectrum

Viscosity. Lowey & Holtzer's (1959) light-scattering and sedimentation results on rabbit myosin and sedimentation results on cod myosin (Connell, 1958*a*) show that the main aggregation mechanism is a side-by-side process. However, viscometry, which ought to be a sensitive indicator of this type of aggregation in such an asymmetric molecule as myosin, suggests that the process cannot be simply an exclusive side-by-side one. For example, the intrinsic viscosity of cod myosin does not change during its extensive aggregation (Fig. 11) and Lowey & Holtzer (1959) found that during the aggregation of rabbit myosin the intrinsic viscosity actually increased. It would therefore appeart hat a full description of aggregation must await further investigation.

Optical rotation. No change in this property of cod myosin could be detected during storage of up to 10 days at 0° but small increases in laevorotation accompany storage for 20 hr. at 23°, e.g. $[\alpha]_D^{23}$ changed in one experiment from $-34{\cdot}2°$ to $-36{\cdot}5°$.

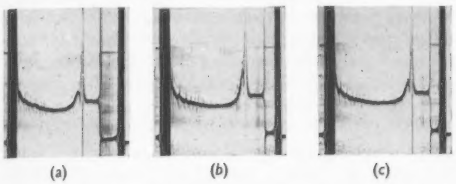

Fig. 8. Ultracentrifuge diagrams showing the effect of (*a*) 0·1 M-ethylene glycol and (*b*) 0·1 M-monothioglycol on the aggregation of cod myosin for 2 days at 0°, pH 7·5, *I* 0·5; (*c*) is the untreated solution. Protein concentration, 0·57%. Sedimentation from right to left; 69 min. after reaching full speed (56 100 rev./min.). Temperature of runs: (*a*) 1·4°; (*b*) 1·5°; (*c*) 1·1°.

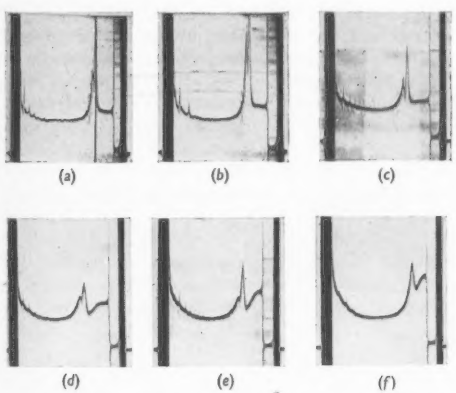

Fig. 9. Ultracentrifuge diagrams showing the effect of urea on the aggregation of cod myosin for 3 days at 0°, pH 7·5, *I* 0·5. (*a*) Untreated; (*b*) 0·3 M-urea-treated; (*c*) untreated; (*d*), (*e*) and (*f*) M-, 2 M- and 4 M-urea-treated respectively. (*a*) and (*b*) are the same preparation (protein concentration, 0·63%). (*c*), (*d*), (*e*) and (*f*) are the same preparation (protein concentration, 0·44%). Sedimentation from right to left: 69 min. after reaching full speed (56 100 rev./min.). Temperature of runs: (*a*) 4·6°; (*b*) 2·0°; (*c*) 3·9°; (*d*) 5·0°; (*e*) 1·3°; (*f*) 0·9°.

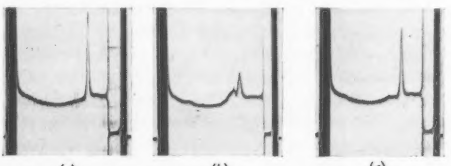

Fig. 10. Ultracentrifuge diagrams of (*a*) cod actomyosin + mM-pyrophosphate, pH 7·5, *I* 0·5; (*b*), (*a*) after 3 days at 0°; (*c*) cod actomyosin used for (*a*) kept 3 days at 0° and then treated with mM-pyrophosphate. Protein concentration, 0·59%. Sedimentation from right to left; 69 min. after reaching full speed (56 100 rev./min.). Temperature of runs: (*a*) 1·5°; (*b*) 3·6°; (*c*) 1·7°.

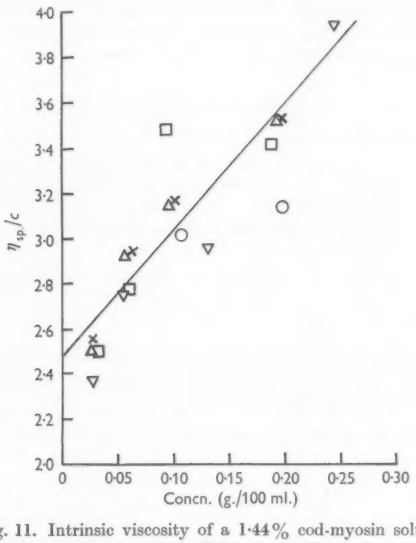

Fig. 11. Intrinsic viscosity of a 1·44% cod-myosin solution during storage at 0°, pH 7·5, *I* 0·5. Time of storage (days): ○, 0; □, 1; △, 2; ×, 3; ▽, 6. $\eta_{sp.}$ is the specific viscosity.

Sulphydryl groups. No changes in either the sulphydryl groups of cod myosin that react freely with *p*-chloromercuribenzoate or the total sulphydryl groups (determined after denaturation in 8M-urea) have been found during aggregation for 6 days at 0°.

Ultraviolet-absorption spectrum. Laskowski, Widom, McFadden & Scheraga (1956) and Scheraga (1957) respectively have proposed that, in insulin and ribonuclease, formation of hydrogen bonds involving tyrosyl groups is revealed by shifts in the ultraviolet-absorption spectra of the proteins. The shifts are observed by differential spectrophotometry, the maximum difference in absorption occurring at 287 mμ. Spectral changes occurring during the polymerization of fibrin also suggests that tyrosyl hydrogen bonds are formed (Scheraga & Laskowski, 1957).

An attempt was made to observe similar shifts during the aggregation of cod myosin by reading aggregated samples against the monodisperse protein adjusted to the same nitrogen content. However, apart from very small differences in absorption at 276 mμ, probably attributable to slight differences in protein concentration, the absorptions of the aggregated and monodisperse samples were identical between 265 and 295 mμ.

Changes in adenosine triphosphatase activity during aggregation

Aggregation at 0° is paralleled by a loss in enzyme activity. The rate of loss of activity is first-order with respect to time up to 3 days' storage but becomes of higher order later. Yasui, Fukazawa, Hashimoto, Kitagawa & Sasaki (1958) found the same behaviour with rabbit myosin or actomyosin. The initial inactivation rate, like that of aggregation, is somewhat erratic; the mean of several experiments in which the myosin was stored at a concentration of 1·2–1·5% gave an inactivation constant [$k_0 = (1/t)/\ln (A_0/A_t)$, where A_0 and A_t are the initial activity and activity after time t respectively] of $0{\cdot}6 \times 10^{-5}$ at 0°. The comparable figure of Yasui *et al.* (1958) for rabbit myosin is $< 0{\cdot}01 \times 10^{-5}$ at 1°.

Although aggregation is accelerated at higher protein concentrations, one experiment (Table 1) indicated that the reverse is true for the inactivation of the enzyme. In other experiments enzyme activity was not lost when cod myosin was aggregated by precipitating and redissolving. Actomyosin retains its activity better on storage than myosin (Table 2).

Table 1. *Inactivation of cod-myosin adenosine triphosphatase during storage at* 0°, pH 7·5, *I* 0·5

Time of storage (days)	Concn. of protein (%)		
	1·20	0·40	0·20
	Percentage of original activity remaining		
1	85	83	63
2	72	50	45
3	54	41	32

Table 2. *Inactivation of adenosine triphosphatase of cod myosin and actomyosin compared at the same protein concentration* (0·23%)

Time represents period of storage at 0°, pH 7·5, *I* 0·5.

Time (days)	Percentage of original activity remaining	
	Myosin	Actomyosin
1	66	85
2	47	80
3	47	71
6	34	64

Tryptic digestion of native and aggregated myosins

Although earlier experiments (Haurowitz, Tunca, Schwerin & Göksu, 1945) had shown that heat coagulation of rabbit myosin had no effect on the rate of its digestion by trypsin, a recent report that an estimate of the amount of 'crystalline' or 'folded' region in a fibrous protein like myosin could be arrived at by measuring its rate of tryptic digestion prompted a similar investigation of cod myosin. Harrington, Hippel & Mihalyi (1959) suggest that digestion can be accounted for by two parallel first-order reactions, the slower involving the 'crystalline' regions and the faster the 'amorphous' regions of the molecule. During the proteolysis of myosin at 6–25° approximately 20% of the bonds are split by the fast reaction whereas at 41° the proportion

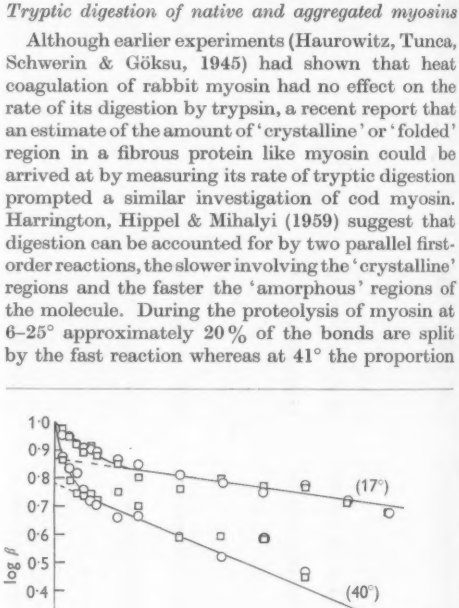

Fig. 12. Tryptic digestion of cod myosin at 17° and 40°. ○, Native myosin; □, myosin denatured by heating at 40° for 30 min. $\beta = 10\ (E_\infty - E_t)$, where E_∞ and E_t are the extinctions of the ninhydrin-treated digests after 24 hr. and t min. of digestion respectively.

is 50%; the conclusion drawn is that the molecule is becoming unfolded ('denatured') at the higher temperature. It was therefore of obvious interest to see if a similar change in the kinetics of tryptic digestion of cod myosin occurred during its aggregation.

The kinetic observations of Harrington *et al.* (1959) have been broadly substantiated, but as shown in Fig. 12 the rate of digestion is the same at any one temperature for aggregated as for monomeric myosin. Aggregation was produced by keeping the myosin at 40° for 30 min. and ultracentrifugal analysis revealed the complete absence of monomer. These results imply that if any such configurational alteration as postulated by Harrington *et al.* (1959) does occur on raising the temperature from 17° to 40°, then it is completely reversible. It is therefore clear that any change in the cod-myosin molecule that accompanies its irreversible aggregation is not revealed by differences in rates of tryptic digestion.

DISCUSSION

The kinetic results just described can be accounted for most simply and at least qualitatively by assuming that the aggregation reaction involves two steps: a first-order step which does not affect the sedimentation coefficient followed by a second- (or higher-) order step. Such a scheme is indicated by the facts that the reaction is concentration-dependent, is apparently first-order at high protein concentrations and of indeterminate order at low concentrations. At the concentrations studied the second step must be more rapid but not enormously more rapid than the first. If the second step were very much more rapid than the first the kinetics would be first-order throughout. If the rates of the two steps were similar deviations from first-order kinetics would appear in the early stages of the reaction.

The two steps of this kinetic scheme can be rationalized in terms of a small configurational alteration in the myosin monomer (M) preceding the step-wise aggregation of the altered molecule (M_D):

$$M \rightarrow M_D \quad (1)$$

$$M_D + M_D \rightarrow 2M_D \quad (2)$$

$$2M_D + M_D \rightarrow 3M_D \text{ etc.}$$

It is not, however, possible to exclude reactions such as

$$M + M \rightarrow 2M$$

and

$$M + M_D \rightarrow M \cdot M_D.$$

A similar two-step scheme has been proposed for rabbit myosin by Lowey & Holtzer (1959), though on very different grounds.

One consequence of this kinetic scheme is that if the measured ultracentrifuge component were all monomer (M) then its rate of disappearance should remain first-order under all conditions. The fact that this is not observed experimentally could be accounted for by assuming that part of the component is due to the presence of the product of step (1), i.e. M_D. In other words the sedimentation behaviour of M_D is similar to that of M. Among the relatively few ultracentrifugal investigations of the denaturation and aggregation of proteins by heat there are two precedents for this suggestion. Heat denaturation of both thyroglobulin (Lundgren & Williams, 1939) and bovine-serum albumin (Warner & Levy, 1958) produces species that have the same sedimentation velocity as the native molecule. Yasui *et al.* (1958) remark on the fact that the monomer peak in the ultracentrifuge picture becomes broader during the aggregation of rabbit myosin. The same phenomenon has been noted with cod, but although it might be due to an alteration in the molecule it could also be explained on the grounds that self-sharpening of the boundary will be less and diffusion greater at the lower myosin concentration.

The first-order reaction (1) is presumably a form of denaturation because the activation energy is high and because extremes of pH or ionic strength and reagents such as urea accelerate aggregation. However, only small increases in laevorotation accompany aggregation, indicating that any configurational disorders are much less than those accompanying urea-denaturation, which produces a fourfold increase in laevorotation. The fact that the numbers of 'free' sulphydryl groups do not change during aggregation is another indication that the degree of unfolding is small.

The rates of aggregation during storage are similar to the rates of enzymic inactivation. For cod myosin, the rate constants for the aggregation and enzymic inactivation of a 1·2–1·5% solution at 0° are 0·45 and $0{\cdot}6 \times 10^{-5}$ sec.$^{-1}$ respectively; values for rabbit myosin at 25° of $0{\cdot}18 \times 10^{-4}$ sec.$^{-1}$ for aggregation (Lowey & Holtzer, 1959) and of $0{\cdot}12 \times 10^{-4}$ sec.$^{-1}$ (interpolated from the data of Yasui *et al.* 1958) and $0{\cdot}13 \times 10^{-4}$ sec.$^{-1}$ (Ouellet, Laidler & Morales, 1952) for enzymic inactivation have been reported. However, the relation between the two processes is not clear since protein concentration has different effects and since precipitated cod myosin retains its enzymic activity.

The present results emphasize that on keeping at low temperatures cod myosin denatures much more readily than the rabbit protein. At 0° inactivation of cod-myosin adenosine triphosphatase proceeds at least 60 times as quickly as that of rabbit myosin. It has also been observed that the ultracentrifuge diagram of rabbit myosin was not

changed by storage for 7 days under the conditions of Fig. 1. The ease with which apparently low-molecular-weight fragments are split off cod myosin, e.g. by urea concentrations as low as 0·3M, may point to a relatively loose internal cohesion of the molecule.

The results of Lowey & Holtzer (1959) and the present ones have given little help in discovering the nature of the bonds holding myosin aggregates together. There is no evidence for sulphydryl interactions being involved. The slight inhibition or reversal of aggregation in concentrated urea solutions suggests that hydrogen bonds might be concerned. The negative results given by the ultraviolet-difference spectra of aggregated versus monomeric cod myosin suggest that tyrosyl groups do not participate in any hydrogen bonding which may occur.

SUMMARY

1. The aggregation of cod-myosin monomer at concentrations of 0·2–1·5% has been studied by following changes in weight-average molecular weight, sedimentation, diffusion, viscosity and optical rotation.

2. The kinetics of the disappearance of monomer have been analysed and can be plausibly accounted for by assuming that the reaction involves two steps: a denaturation stage followed by a relatively rapid stepwise side-to-side aggregation.

3. Additional evidence for a denaturation stage has come from a study of the reaction at different temperatures, conditions of pH and ionic strength, from the effects of added substances and from an examination of the concurrent enzymic inactivation.

4. Aggregation, which under extreme conditions is often accompanied by a dissociation of the molecule, cannot be explained on the basis of sulphydryl–disulphide interactions.

Mr P. F. Howgate assisted in the experiments described in this paper. The work described was carried out as part of the programme of the Department of Scientific and Industrial Research.

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Intracellular Distribution of Carbonic Anhydrase

BY R. KARLER AND D. M. WOODBURY*
Department of Pharmacology, University of Utah College of Medicine, Salt Lake City, Utah, U.S.A.

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The presence of carbonic anhydrase in various mammalian tissues has been recognized for many years. The only known function of the enzyme is catalysis of the reaction

$$CO_2 + H_2O \rightleftharpoons H_2CO_3.$$

In certain organs, such as stomach, kidney and pancreas, it appears that carbonic anhydrase is involved in adjusting the rate of a steady state of production of carbon dioxide or carbonic acid to specific functional demands. The necessity for an increased rate of the above reaction is most clearly understood for the example of elimination of

* Scientist of the National Neurological Research Foundation.

carbon dioxide. The relatively short period of time in which blood remains in capillaries, both in systemic and in the lung, would not be adequate for elimination of carbon dioxide in the absence of carbonic anhydrase. Hence, in this particular example, the function of the enzyme has been clearly demonstrated to be an integral part of the more general function of respiration.

The role of carbonic anhydrase in tissues such as liver and brain, however, which generally are not associated with recognizable functions involving carbon dioxide or acid secretion is unknown. In view of this fact a study of the intracellular distribution of the enzyme in several different tissues was conducted in order to obtain additional descriptive information, which in turn might be useful in developing an understanding of the role of the enzyme.

Available data on the intracellular locus of carbonic anhydrase are limited. Datta & Shepard (1959) reported that the distribution of the enzyme in rat liver and kidney was restricted to the soluble fraction, whereas Karler & Woodbury (1959) presented preliminary evidence which indicated that the intracellular distribution was more complicated. Our studies indicated that carbonic anhydrase was present, not only in the soluble fraction, but also in particulate matter which corresponded to mitochondria.

The work described here represents continued investigation of the intracellular distribution of the enzyme in the rat tissues, cerebral cortex, kidney, liver, salivary glands and thyroid. The results support the previous report that generally carbonic anhydrase is distributed intracellularly between soluble and particulate fractions.

METHODS

The various tissues studied were taken from male, Sprague–Dawley rats which weighed approx. 250 g. Tissues were perfused *in vivo* by the following procedure: animals were anaesthetized with ether and a needle and syringe were used to withdraw as much blood as possible from the dorsal aorta at the junction of the common iliacs. The needle was held in position by a haemostat. After the withdrawal of blood, 10 ml. of 0·25 M-sucrose solution was injected and 10 ml. of diluted blood removed. This perfusion procedure was repeated three times. With cerebral cortex, kidney, liver and salivary glands approx. 1–2 g. of tissue was taken for homogenization. Thyroid determinations, however, involved about 100 mg. of tissue, which was collected by pooling glands from 10 rats. Perfused tissues were homogenized in 10 vol. of 0·25 M-sucrose solution with a glass–Teflon type of tissue grinder (A. H. Thomas Co.), 15–20 strokes at 350 rev./min. for about 3 min. being used. The homogenization was performed in an ice bath and all subsequent manipulation of tissue fractions was in a cold room (5°).

The differential-centrifuging procedure used for the determination of the intracellular distribution of the enzyme corresponded in general to that proposed by Hogeboom & Schneider (1955). Tissue homogenates were fractionated into total particulate matter and soluble fraction by centrifuging at 100 000 *g* for 30 min. The particulate fraction was washed once by resuspension and centrifuging again in 0·25 M-sucrose solution. The homogenization and centrifuging procedures were repeated with different suspending media. In addition to 0·25 M-sucrose solution, 0·88 M-sucrose solution (Hogeboom & Schneider, 1948) and 0·25 M-sucrose–7·3% polyvinylpyrrolidone (General Aniline and Film Corp.) solution (Novikoff, 1957) were also employed.

Homogenates of cerebral cortex were fractionated in more detail by the following procedure. A nuclear fraction was collected by centrifuging at 600 *g* for 10 min.; a mitochondrial fraction was obtained by continuing the centrifuging at 10 000 *g* for 25 min.; the final particulate fraction, the microsomes, consisted of particles removed by centrifuging the remaining suspension at 100 000 *g* for 30 min. Each particulate fraction was subjected to one washing. The soluble fraction represented the supernatant obtained from the differential centrifuging of the particulate fractions. Kidney homogenates were also fractionated but the mitochondrial fraction was collected by centrifuging the nuclear-free homogenate at 20 000 *g* for 10 min.

Carbonic-anhydrase activity was measured by the manometric method described by Roughton & Clark (1951). Enzymic activities were determined in samples that were frozen for storage and subsequently thawed for assay, and in fresh samples, which were assayed as soon as possible after isolation. Except for initial experiments, all assays were performed on fresh samples.

Enzymic activities of tissue homogenates were expressed in terms of units as defined by Meldrum & Roughton (1934). Units are calculated from the expression $(t_u - t_c)/t_c$, in which t_u represents the time required for the uncatalysed reaction to produce the second quarter of the total manometric displacement and t_c represents the time required for the catalysed reaction to produce the same quarter of the reaction.

The enzymic activities of subcellular fractions were expressed as a percentage of the total enzymic activity of the original homogenate by determining a standard curve of homogenate activity (in terms of rates) at various concentrations. The expression of recoveries for the fractionation studies represents the sum of the activities of the fractions in terms of homogenate activity.

RESULTS

Carbonic-anhydrase activity in particulate and soluble fractions

Preliminary attempts to determine the intracellular distribution in the two major fractions were quantitatively unsuccessful because of an inability to obtain adequate enzymic recoveries. Initially, only 60–70% of the total activity was recoverable. At this time samples were frozen for storage before they were assayed, but it was subsequently observed that freezing and thawing significantly affected the activity of the soluble fraction. The data are given in Table 1.

As is indicated, the total activities in the homogenate and particulate fractions were not strikingly affected by freezing. However, the enzymic activity in the soluble fraction, which represented 67 % of the total activity before freezing and only 46 % after freezing, was greatly decreased by the freezing and thawing procedure.

The results obtained when the fractions were assayed immediately after preparation are given in Table 2. With the exception of the thyroid, the soluble fraction from the other tissues contained about 75–85 % of the total enzymic activity. The remaining 15–25 % of the activity was recovered in the particulate fraction.

The results for the thyroid indicated that, under the conditions of these experiments, most if not all of the activity appeared in the soluble fraction. No activity was detected at any time in the particulate fraction.

The distribution results obtained with 0·88 M-sucrose and 0·25 M-sucrose–7·3 % polyvinylpyrrolidone media as compared with the conventional 0·25 M-sucrose solution are cited in Table 3. It can be seen from this table that the distribution of carbonic-anhydrase activity between total particulate and soluble fractions remains practically the same in the three different fractionation media.

The results in Table 3, however, raise an interesting problem. From comparison of the total enzymic activity recovered with comparable deter-

Table 1. *Effect of freezing on the recovery of carbonic-anhydrase activity from cellular fractions of cerebral cortex*

Cortical tissue was homogenized in 10 vol. of 0·25 M-sucrose soln. and the particulate fraction was separated from the soluble fraction by centrifuging at 100 000 *g* for 30 min. Samples were first assayed immediately after preparation and then stored at −20° for 24 hr. Samples were subsequently thawed at room temperature and assayed again. Duplicate values given represent the results obtained from two experiments.

	Percentage of activity of homogenate	
	Before freezing	After freezing
Total homogenate activity	100 (280, 290 units)	100 (296, 312 units)
Particulate fraction	21, 25	21, 19
Soluble fraction	63, 71	41, 51
Total recovery	84, 96	62, 70

Table 2. *Approximate intracellular distribution of carbonic-anhydrase activity between particulate and soluble fractions*

Tissues were homogenized in 10 vol. of 0·25 M-sucrose soln. and the particulate fraction was separated from the soluble fraction by centrifuging at 100 000 *g* for 30 min. Samples were assayed immediately after preparation. The number of experiments on each tissue is given in parentheses; the enzymic activities represent average values. Values for total enzymic activities are to the nearest hundred, except for the thyroid, which is to the nearest ten. The percentage total activity recovered in soluble and particulate fractions is to the nearest 5 %. The percentage recovered values represents recoveries for the individual experiments.

Tissue	Total enzymic activity (units/g. wet wt.)	Recovered enzymic activity (%)		Total enzymic activity recovered (%)
		Soluble fraction	Particulate fraction	
Submaxillary gland (2)	2400	75	25	88, 100
Kidney (2)	1000	80	20	77, 87
Cerebral cortex (6)	300	75	25	86, 90, 94, 96, 85, 94
Liver (2)	300	85	15	84, 96
Thyroid (3)	60	100	—	76, 80, 94

Table 3. *Influence of various homogenization media upon the intracellular distribution of carbonic-anhydrase activity in cerebral cortex*

Fractionation procedures are described in the Methods section. Duplicate values given represent the results obtained from two experiments with each medium.

Medium	Total activity (units)	Percentage of total enzymic activity		Percentage recovery
		Particulate fraction	Soluble fraction	
0·25 M-Sucrose	70, 73	37, 45	49, 54	86, 99
0·25 M-Sucrose–7·3 % polyvinylpyrrolidone	72, 78	39, 42	49, 57	88, 99
0·88 M-Sucrose	98, 111	40, 45	55, 68	95, 113

minations reported in Tables 1 and 2, it can be seen that the total enzymic activity (units/g.) decreased from about 300 to less than 100. Further comparison of these tables indicates that relative changes in the intracellular distribution also occurred. As shown in Table 3, about 40% of the enzymic activity is associated with the particulate fraction, whereas the corresponding fraction in the previous tables contained only 25% of the total activity. At the present time these changes in total activity and distribution are not understood.

Intracellular distribution of carbonic anhydrase in rat cerebral cortex and kidney

The results obtained by differential centrifuging are given in Table 4. With cerebral cortex, the experimental results indicated that the soluble fraction contained approximately 75% of the total enzymic activity, whereas the mitochondrial fraction itself possessed 20% of the activity. The remaining 5% of activity in cortical homogenates was recovered in the nuclear fraction.

The intracellular distribution of carbonic anhydrase in the varicus kidney fractions appeared similar to that obtained for cerebral cortex. The majority of the total activity (80%) again appeared associated with the soluble fraction, and the activity in the particulate fractions was also greatest in the mitochondria (10%). The kidney nuclei and microsomes each contained approximately 5% of the total activity.

Effect of solubilization procedures upon the carbonic-anhydrase activity associated with mitochondria of cerebral cortex

The data in Table 5 represent the effects upon the insoluble carbonic anhydrase observed after exposing mitochondria to water, to repeated freezing and thawing and to digitonin. The results demonstrated that the enzymic activity associated with mitochondria isolated in a sucrose medium (control value) was not solubilized by any of the experimental procedures employed, that is, the enzymic activities of the mitochondrial washes remained zero even after the various treatments.

Such observations tend to indicate that the enzyme is not merely contained in solution within mitochondria, but that the enzyme is intimately associated with the mitochondrial membrane.

DISCUSSION

The data presented on the intracellular distribution of carbonic anhydrase provide evidence for the existence of the enzyme in both the soluble and particulate components of cells. The more detailed studies which were performed on cerebral cortex and kidney indicate that the enzymic activity of the particulate matter is generally associated with the mitochondrial fraction.

Any intracellular-distribution study involves several technical problems. The fact that red blood cells contain carbonic anhydrase and hence are capable of contaminating the true tissue activity is in itself an additional complication. Some studies in the past on tissue carbonic anhydrase have taken this into account by correcting levels of enzymic activity for the presence of blood. This type of correction was not felt to be adequate for intracellular-distribution studies because rat blood contains, in addition to significant amounts of carbonic anhydrase in red blood cells, a potent, carbonic-anhydrase inhibitor in serum or plasma,

Table 4. *Intracellular distribution of carbonic anhydrase in rat cerebral cortex and kidney*

Fractionation procedures are described in the Methods section. The activities in cerebral-cortex fractions represent the average of four experiments; the activities in kidney fractions represent the average of two experiments. The averages (%) for all fractions are expressed to the nearest 5%.

	Percentage of total activity recovered	
Cellular fraction	Cerebral cortex	Kidney
Nuclei	5	5
Mitochondria	20	10
Microsomes	—	5
Soluble fraction	75	80
Percentage recovery	95	90

Table 5. *Effect of solubilization procedures upon carbonic-anhydrase activity of mitochondria of cerebral cortex*

Control indicates a mitochondrial sample suspended in 0·25 M-sucrose solution for 1 hr. at 5°; water indicates that a sample was suspended in water for 1 hr. at 5°; water, frozen and thawed, indicates that a sample was suspended in water and frozen and thawed three times; digitonin indicates that a sample was suspended in 1% digitonin (Cooper & Lehninger, 1956) for 1 hr. at 5°. After these treatments the suspensions were centrifuged at 100 000 *g* for 30 min. and the carbonic-anhydrase activity was determined in the pellet and supernatant. Results represent values obtained from two experiments for each treatment. The activity of mitochondria after treatment represents the average of the duplicate experiments.

	Activity of supernatant (units)		Activity of mitochondria after treatment (units)
Treatment	Before treatment	After treatment	
Control	0	0	40
Water	0	0	40
Water, frozen and thawed	0	0	50
Digitonin	0	0	65

as reported by Booth (1938). In view of the complexity of rat blood with respect to carbonic-anhydrase activity, the technique of repeated perfusions through the dorsal aorta was introduced in an attempt to remove as completely as possible the contaminating blood. The perfusion procedure as described in the Methods section reduced tissue-carbonic-anhydrase activity to a constant level as determined by the observation that additional perfusions did not lower the enzymic activity in the various tissues. Furthermore, the report by Datta & Shepard (1959) that contaminating red cells survived the homogenization and were isolated with the particulate fraction of cells was confirmed. In our studies the few red blood cells which remained after perfusion were isolated with the nuclear fraction, as could be determined by observation of this pellet. The fact that the nuclear fraction contained only a small percentage of the total enzymic activity was taken as additional evidence for the completeness of the perfusion.

In addition to the problem of blood contamination, other technical problems common to all fractionation studies exist. The presence of carbonic anhydrase in both particulate and soluble fractions immediately raises two general considerations about the validity of such a distribution pattern. It is conceivable that the activity of the soluble fraction is a consequence of a leaching out or an elution of the enzyme from subcellular particles, and also the possibility exists that the activity associated with the particulate fraction is due to contamination by the soluble fraction.

Experiments were performed to test the possibility that the activity in the soluble fraction may be derived from particles. Such an elution phenomenon was reported for catalase, whose intracellular locus was ultimately restricted to particles, as shown by Greenfield & Price (1954) with the use of a sucrose–polyvinylpyrrolidone suspending medium. Various media have been employed by many investigators in attempts to preserve the morphological and biochemical integrity of isolated mitochondria.

The experiments cited in this paper include the fractionation of tissue in three different suspending media: (i) 0·25M-sucrose solution; (ii) 0·25M-sucrose solution–7·3% polyvinylpyrrolidone; (iii) 0·88M-sucrose solution. The results from such studies indicated that the distribution of enzymic activity between soluble and particulate fractions remained constant, regardless of the suspending medium. Therefore on the basis of these experiments it does not appear that the carbonic-anhydrase activity in the soluble fraction is derived from morphological or biochemical disturbances of isolated mitochondria. This conclusion was also reached by Datta & Shepard (1959).

The evidence which argues against the possibility that the enzymic activity associated with particulate matter is due to contamination rests on the observations that repeated washings of the particulate matter yielded decreasing activity in the washes. For practical purposes one washing yielded a particulate fraction with a constant activity. This fact tends to indicate that such contamination as exists is distinct from a more fixed enzymic activity. The same observation was reported by Datta & Shepard (1959), who maintained that 20–30% of the total activity in their preparations was associated with particulate matter. However, they attributed the presence of activity in particulate fractions to contamination, despite the fact that activity persisted even after repeated washings. No explanation was offered for the inability to wash out the activity.

Additional support for the presence of carbonic anhydrase in particles is provided by procedures designed to disrupt mitochondria and then to attempt physically to separate enzymic activity from mitochondrial residue. Such experiments demonstrated that the insoluble nature of the enzyme was not affected by treatment with water, by repeated freezing and thawing and by exposure to digitonin. Hence it is concluded that the enzyme is bound inextricably to the mitochondrial membrane, as has been reported for certain insoluble enzymes of the Krebs cycle (Hogeboom, 1955).

Another means of contaminating particles must also be considered; namely, the activity associated with the particulate fraction may be the result of an adsorption arising from a juxtaposition with the soluble enzyme as a consequence of homogenization. In this manner, microsomes have been shown to be capable of adsorbing ribonuclease (Schneider & Hogeboom, 1952), fumarase (Kuff, 1954) and haemoglobin (Paigen, 1956). Attempts to associate adsorption characteristics with mitochondria have not been successful (Schneider & Hogeboom, 1952; Beinert, 1951). From the experiments cited on carbonic anhydrase such a phenomenon cannot be ruled out.

The results of the experiments reported above corroborate our earlier observation of a bimodal intracellular distribution for carbonic anhydrase. The problem of the physiological role of this enzyme in such tissues as the brain still exists.

SUMMARY

1. Various tissues of the rat were studied by differential centrifuging for their intracellular distribution of carbonic anhydrase.

2. Cerebral cortex, kidney, liver and salivary glands appeared to contain enzymic activity in both particulate and soluble fractions. A majority

of the activity was associated with the soluble fraction.

3. The activity of the thyroid gland, however, was restricted to the soluble fraction.

4. More detailed fractionation studies of cerebral cortex and kidney indicated that the particulate matter associated with enzymic activity corresponded in general to the mitochondrial fraction.

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The Metabolism of Phenolic Antioxidants

2. THE METABOLISM OF BUTYLATED HYDROXYANISOLE IN THE RAT*

BY B. D. ASTILL, D. W. FASSETT AND R. L. ROUDABUSH
Laboratory of Industrial Medicine, Eastman Kodak Company, Rochester, New York, U.S.A.

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Commercial 'butylated hydroxyanisole' consists of a mixture of 15% or less of 2-*tert.*-butyl-4-hydroxyanisole (isomer A) and 85% or more of 3-*tert.*-butyl-4-hydroxyanisole (isomer B). The introduction of butylated hydroxyanisole as a fat-stabilizer about 10 years ago, and its subsequent extensive use in edible fats, has been accompanied by studies of its acute and chronic toxicity (e.g. Graham, Teed & Grice, 1954). Wilder & Kraybill (1948), found none of the substance, other than questionable trace amounts in depot and carcass fat of rats receiving 3% of butylated hydroxyanisole in the diet over a prolonged period. Studies on the storage of the compound in dogs given daily doses of 0·3, 30 and 100 mg./kg. for more than a year (Hodge & Fassett, unpublished work) also failed to reveal any butylated hydroxyanisole in perirenal, omental or subcutaneous fat or in the brain, liver and kidney.

The metabolism of butylated hydroxyanisole has been studied in the rabbit by Dacre, Denz & Kennedy (1956), who found that an oral 0·5 g. dose was excreted as glucuronide (60%), ethereal sulphate (12%) and free phenols (4% of the dose). This dosage is very large compared with human intake. The ingestion of phenolic antioxidants as a result of their use in fat stabilization is probably less than 0·1 mg./kg. It is known that variations in dose may alter the proportions of metabolites excreted, where more than one metabolic pathway is available (cf. phenol; Bray, Thorpe & White, 1952). To justify extrapolation from experimental doses to the usage dose, it is desirable to study the pattern of metabolism with a variety of doses, including the lowest possible so that any unsuspected changes in metabolic pattern may be detected. This paper deals in this way with the metabolism in the rat of butylated hydroxyanisole and its component isomers.

* The paper by Astill, Fassett & Roudabush (1959) is to be regarded as Part 1.

METHODS AND MATERIALS

Animals, diets and dosage. Male and female albino rats of Sprague–Dawley strain, average wt. of 128 rats 321 g. (224–443 g.), were fed with Purina Chow and kept singly or

in pairs in metabolism cages. Butylated hydroxyanisole, isomer A or isomer B was administered by oral injection in one of the media shown in Table 5. Control values for metabolites were established by analysis of the pooled urine collected for 1 day before dosing. Urines were analysed immediately or kept frozen until analysis. The total faeces in Expts. 14 and 16 were collected and analysed for unabsorbed butylated hydroxyanisole.

Materials. Butylated hydroxyanisole, m.p. 54–58°, b.p. 264–270° at 733 mm. Hg, isomer A, m.p. 62·5–64·0°, and isomer B, m.p. 64·5°, were kindly supplied by the Tennessee Eastman Co., Kingsport, Tenn., U.S.A., to whom we are indebted for the physical data. Chromatographic analysis showed that isomer A contained 3–5% of *p*-methoxyphenol. The following were purchased: 2:6-dichloroquinonechloroimide, 2:2′-dipyridyl, *tert.*-butylquinol, naphtharesorcinol, D-glucuronolactone (Eastman Organic Chemicals, Rochester, N.Y.), β-glucuronidase (ox-liver; Ketodase, Warner–Chilcott, N.Y.), Safranine bluish (3-amino-7-dimethylamino-5-phenylphenazinium chloride, Allied Chemical and Dye Corp., N.Y.).

Paper chromatography. Table 1 records R_F values, colour reactions and solvent systems: strips of Whatman no. 1 paper were used in the descending method. Isomer A (2–3 μg.) and isomer B (1 μg.) were visually detectable with spray reagents A and C. R_F values of metabolites were determined on the purest available specimens. Chromatograms were scanned at 225 or 275 mμ with an attachment inserted in the cell-holder space of a Beckman model DU spectrophotometer (Table 2). Chromatograms were scanned quantitatively after colour development with a Photovolt Electronic Densitometer model 525 (Photovolt Corp., 95 Madison Avenue, N.Y.).

Estimation of butylated hydroxyanisole on chromatograms. From 5 to 40 μg. of each isomer was chromatographed in solvent II for 2 hr. or solvent III for 1 hr. The dried papers were sprayed with (i) reagent A, and absorbing areas corresponding to butylated hydroxyanisole were scanned at 445 mμ, or (ii) reagent C (Gibbs's Reagent; Mahon & Chapman, 1951), dried and scanned at 595 mμ. Elution of spots located by co-chromatography gave low recoveries with reagent C spectrophotometrically. Results, recorded graphically by plotting extinction against distance along the paper and integrating, showed a linear relation between total extinction of a spot and amount chromatographed. Considerable variation from experiment to experiment was found: thus 20 μg. of isomer B, sprayed with reagent C, gave a mean area in thirteen separate experiments of 249 (212–290) mm.², although good replicates were obtained on co-chromatography. A known amount of the isomer to be determined was co-chromatographed in each determination.

Examination of urine

Paper chromatography. Experimental and control urines (10 μl. each) were compared as in Table 1. Only isomers A and B and a metabolite of isomer A, compound H, could be detected in experimental urines. Scanning of chromatograms in ultraviolet light revealed no metabolites because of absorption by substances of natural origin. Elution and estimation of metabolites located by co-chromatography gave low recoveries.

Ethereal sulphates. The total and inorganic sulphate content was estimated either by Folin's gravimetric or by Sperber's turbidimetric method (Bray & Thorpe, 1954).

Free isomers A and B. Urine (5 or 10 ml.) was gently shaken with ether (60 ml.) for 16–24 hr. The separated ether extract was evaporated to dryness at a temperature below 60–70°. A portion of a solution of the residue in ethanol (0·5 ml.) was chromatographed and the free isomer content was estimated chromatographically. Recoveries from control urine (5 ml.) of added isomer A (0·10–0·72 mg.) were 94±4%, and of added isomer B (0·10–0·78 mg.) were 94±3%. The ethereal sulphates of isomers A and B did not interfere with this estimation, as shown by separate experiments in which 0·15 μmole of isomer A and 0·1 μmole of isomer B were recovered from control urine (10 ml.) to which the corresponding ethereal sulphates (34 μmoles) had been added. In view of the known instability of butylated hydroxyanisole in acid solution (Dacre *et al.* 1956) the usual estimation of combined phenols by acid hydrolysis followed by ether extraction was omitted.

For glucuronides. Total glucuronides were estimated by a modified naphtharesorcinol method (Astill *et al.* 1959), and in low-dose studies by the following procedure. The glucuronide gum obtained by precipitation of the insoluble lead salts at pH 9·5 (as in the procedure of Kamil, Smith & Williams, 1951) from 10 ml. of urine was redissolved in water. A portion, giving an estimated maximum hydrolysis concentration of 0·4 mM of required glucuronide, was added to 5 ml. of 0·2 M-sodium acetate buffer, pH 4·6, and 2 ml. of β-glucuronidase (≡ 10^4 Fishman units, Talalay, Fishman & Huggins, 1946). The mixture was incubated for 16–24 hr. at 37·4°, extracted with ether (2 × 50 ml.) and the extract evaporated and analysed chromatographically for the required isomers. Ether extracts of hydrolysates of similarly treated control urines gave unexpectedly high extinctions at 600 mμ with Gibbs's reagent. This was caused by substances, absent from experimental ether extracts, which gave intense colours with reagent C at R_F 0·4–0·6 (solvent II). Recoveries by the enzymic and chromatographic methods of the glucuronide of isomer B (2·3–19·0 mg., 0·14–0·36 mM in the hydrolysis) added to urine (10 ml.) were 67±4% in nine experiments. Recoveries of this glucuronide (5–20 mg., 0·27 mM in the hydrolysis) from urine without a preliminary lead-salt separation were 24–96% and varied inversely as the volume of urine (2·0–0·4 ml.) in the hydrolysis.

The lead salts of the ethereal sulphates of both isomers precipitated with those of the glucuronides at pH 9·5, but interference with the glucuronide estimation was negligible. Non-enzymic hydrolysis of the ethereal sulphate of isomer A (1·1 mg.) was 1·7% and of that of isomer B (0·95 mg.) was 1·3%. β-Glucuronidase was without action on either sulphate.

Examination of faeces

Total faeces were analysed chromatographically by the ferric chloride–2:2′-dipyridyl method: the excess of reducing power over control faeces was expressed as butylated hydroxyanisole (Astill *et al.* 1959).

RESULTS

Excretion of metabolites by control animals. The average daily excretion by undosed rats was 11·9 mg. (4·8–20·4 mg.) of ethereal sulphates as butylated hydroxyanisole [ethereal sulphate/in-

Table 1. *R_F values and colour reactions of isomers A and B and their metabolites*

Whatman no. 1 filter paper was used throughout in the descending technique. Solvents were: I, organic phase of freshly mixed butanol–acetic acid–water (4:1:5, by vol.); II, organic phase of benzene–acetic acid–water (2:2:1, by vol.); III, organic phase of butanol–formic acid–light petroleum, b.p. 66–75° (1:10:10, by vol.). (1) After 10–15 cm. development; (2) after 35–40 cm. development. Spray reagents were: A, aq. 1% (w/v) silver nitrate mixed with 1 part of aq. 3N-NH_3 soln.; B, equal volumes, freshly mixed, of 1% (w/v) sulphanilic acid in 3N-HCl and aq. 5% (w/v) $NaNO_2$; the dried paper was sprayed with aq. 2% (w/v) Na_2CO_3; C, ethanolic 0·05% (w/v) 2:6-dichloroquinonechloroimide; the dried paper was sprayed with aq. 2% (w/v) sodium borate, or, D, with aq. N-NH_3 soln.; E, N-HCl; the dried paper was heated at 70° for 3 min. and treated as in D. – indicates that no colour reaction was given.

	R_F			Colours given by reagents				
Substance	Solvent I	Solvent II	Solvent III	A	B	C	D	E
Isomer A	0·96	0·92	(1) 0·90 (2) 0·82	Blue–brown	Orange	Blue–grey	Dark blue	–
Isomer B	0·96	0·94	(1) 0·90 (2) 0·90	Blue–black	Orange–red	Blue–purple	Purple	–
2:5-Di-*tert.*-butyl-4-hydroxyanisole	0·95	0·94	(1) 0·94	Weak brown	Weak orange	Pink	–	–
p-Methoxyphenol	0·95	0·80	(1) 0·32	Brown	Red	Blue	.	.
Ethereal sulphate of isomer A	0·82	0·10	(1) 0·00	–	–	–	–	Blue–grey, fades to grey
Ethereal sulphate of isomer B	0·79	0·07	(1) 0·00	–	–	–	–	Bright blue, fades to pink
Glucuronide of isomer A	0·90	0·00	(1) 0·00	–	–	–	–	Very weak blue
Glucuronide of isomer B	0·89	0·00	(1) 0·00	–	–	–	–	Very weak purple
Compound H	0·83	0·00	(1) 0·00	–	Orange	Weak purple	Mauve	Yellow
tert.-Butylquinol	0·97	.	(1) 0·50	Black	Purple	Blue	Blue–purple	.

organic sulphate ratio 0·137 (0·068–0·201)], determined gravimetrically on fourteen groups each of eight rats, and, as determined by the procedures for low doses, on eight groups each of eight rats, 0·15 mg. (0–0·32 mg.) of glucuronides and 0·05 mg. (0–0·11 mg.) of free phenols, as isomer B.

Tables 3–5 record the excretion of metabolites in dose studies.

Metabolism of isomer A. Administration of a single dose of 0·4 g. of isomer A/kg. caused an increase in urinary ethereal sulphate output sufficient to account for 72 % of the dose, and a much smaller increase in glucuronide output. About 2 % of the dose was recovered from the urine unchanged. Chromatography revealed a minor metabolite, compound H. Repeated doses of 0·5 g./kg. depressed ethereal sulphate output and considerably elevated glucuronide output, the over-all conjugate recovery being slightly lowered. Faecal excretion in repeated-dose studies was 0·2 % of the total dose. In Expt. 15, 0·6 % of the dose was excreted as the impurity *p*-methoxyphenol and 2·6 % as its glucuronide, estimated enzymically and chromatographically.

Metabolism of isomer B. After a dose of 0·4 g./kg., increased glucuronide excretion accounted for 57–71 % and increased ethereal sulphate output for 15 % of the dose; 4 % was excreted unchanged. No minor metabolites were detected. Repeated doses of 0·5 g./kg. lowered the proportion of the dose excreted conjugated. Faecal excretion was 0·3 % of the dose.

Metabolism of butylated hydroxyanisole. A single dose of 0·4 g./kg. was metabolized in a similar manner to a single dose of 0·4 g. of isomer B/kg.; no increase in ethereal sulphate output was caused by the 15 % of isomer A in butylated hydroxyanisole. Repeated doses of 0·5 g./kg. depressed the proportion of the dose excreted conjugated. With single doses of 2–100 mg./kg., consistently high recoveries of the dose from the urine were found. Excretion of butylated hydroxyanisole was at a maximum on the first day after dosing, and the time for complete excretion of the dose decreased as the dose decreased. The glucuronide conjugation remained the principal metabolic pathway at these doses, accounting for 76 % (69–92 %) of the dose. The percentage excretion of unchanged butylated

Table 2. *Absorption spectra of some derivatives of isomers A and B*

Solvents were (*a*) ethanol and (*b*) water.

Compound	$\lambda_{max.}$	log ϵ	$\lambda_{max.}$	log ϵ
Isomer A (*a*)	225	3·79	288	3·56
Potassium 3-*tert.*-butyl-4-methoxyphenylsulphate (*b*)	225	3·85	276	3·36
Methyl(triacetyl-3-*tert.*-butyl-4-methoxyphenyl-β-glucosid)-uronate (*a*)	225	3·94	280	3·34
3-*tert.*-Butyl-4-methoxyphenyl-β-glucosiduronic acid (*b*)	223	3·89	280	3·36
Isomer B (*a*)	226	3·90	289	3·56
Potassium 2-*tert.*-butyl-4-methoxyphenylsulphate (*b*)	224	3·85	278	3·33
2-*tert.*-Butyl-4-methoxyphenyl-β-glucosiduronic acid (*b*)	224	3·92	280	3·39
Compound H (*b*)	225	—	275	—

Table 3. *Urinary excretion of butylated hydroxyanisole, isomer A and isomer B and their metabolites by rats for 5 days after a single dose of 0·4 g./kg.*

Results are expressed as % of dose fed. Compounds were injected by gastric tube as (*a*) a 50 % (w/v) solution in propylene glycol or (*b*) a 10 % (w/v) suspension in pea soup (Strained Baby Foods, Beech-Nut Co., Syracuse, N.Y.) diluted with an equal volume of water into each rat in a group of four (Expts. 1–6) or eight (Expt. 7) and urine was collected daily. Values were obtained daily on the pooled daily output of each group. Unless specified otherwise sulphates were measured gravimetrically (Bray & Thorpe, 1954) and glucuronides by the modified naphtharesorcinol method (Astill, Fassett & Roudabush, 1959).

Compound	Expt.	Medium	Average dose (g./rat)	Free butylated hydroxy-anisole	Glucuronide	Ethereal sulphate	Total accounted for
Isomer A	3	*a*	0·09	2	11	73*	86
	4	*b*	0·12	1	4†	61	66
	5	*b*	0·13	2	10†	87	99
Isomer B	2	*b*	0·11	3	71	16	90
	6	*a*	0·11	4	57	15*	76
Butylated hydroxyanisole	1	*a*	0·08	3	61	16*	80
	7	*a*	0·10	7	82	11	100

* Estimated by Sperber's method (Bray & Thorpe, 1954). † Estimated enzymically.

hydroxyanisole in the urine decreased as the dose increased.

Metabolites of isomers A and B

The glucuronide of isomer A could not be isolated, but its crude methyl-triacetyl derivative, after separation on acid-washed alumina, was converted into 3-*tert.*-butyl-4-methoxyphenyl-β-glucuronamide, identical with a synthetic specimen. The ethereal sulphate of isomer A was isolated from the glucuronide gum as its Safranine bluish salt (Dodgson, Rose & Spencer, 1955) and was converted by cation exchange into potassium 3-*tert.*-butyl-4-methoxyphenyl sulphate; both salts were identical with synthetic specimens. Compound H was formulated as a 2 (or 3)-*tert.*-butyl-4-hydroxyphenyl sulphate, since mild acid hydrolysis of a chromatographically pure specimen yielded only SO_4^{2-} ions and *tert.*-butylquinol, identified spectroscopically and chromatographically. The glucuronide of isomer B was isolated via its barium salt (Dacre *et al.* 1956) from the glucuronide gum. No ethereal sulphate of isomer B was isolated, although its Safranine bluish salt was readily prepared. No other metabolites of isomers A and B were detected.

EXPERIMENTAL

Melting points are uncorrected.

Examination of urine for metabolites

Metabolism of butylated hydroxyanisole. Urine (860 ml.) from Expt. 18, total dose 15·5 g., was clarified by centrifuging and the sediment reserved; the liquid was extracted with ether (2 × 800 ml.) by gentle shaking for 2 hr., and the ether extract reserved. Glucuronide gums were obtained in the usual way by precipitation with lead acetate soln. at pH 4·5 and 9·5 (Kamil *et al.* 1951).

Sediment. The dry sediment yielded an ether-soluble fraction (0·25 g.), which two-dimensional chromatography showed to be isomer A mixed with a trace of isomer B.

Ether extract. The semi-crystalline residue yielded on distillation at 35 mm. Hg and 150–155° a crystalline solid (0·41 g.), shown chromatographically to be isomer B [R_F in solvent III (2), 0·90] with a trace of isomer A [R_F in solvent III (2), 0·85].

Glucuronide gum (pH 4·5). The aqueous solution (500 ml.) mixed with saturated barium acetate solution (8 ml.) was slowly concentrated *in vacuo*. An inorganic precipitate was discarded. The filtrate yielded a gum which was redissolved, freed from Ba^{2+} ions with CO_2, the filtrate evaporated and the residue extracted with ethanol and the ethanol extract concentrated to a gum (4·9 g.). This was shown, by hydrolysis of 0·017 g. with β-glucuronidase, and chromatography of the ether extract of the hydrolysate, to contain 0·26 g. of mixed isomer A and B glucuronides as butylated hydroxyanisole, and no glucuronides other than those normally found in this fraction. The total SO_4^{2-} ion content of the gum, measured gravimetrically, was equivalent to 0·03 g. of ethereal sulphates as butylated hydroxyanisole.

Table 4. *Urinary excretion of butylated hydroxyanisole and its metabolites by rats after a single dose*

Results are expressed as % of dose fed. Each rat in a group of four (Expts. 12, 13) or eight (Expts. 8–11) received butylated hydroxyanisole by gastric tube in diluted pea soup. Analyses were performed on the pooled daily output of all rats in a group, collected until no further metabolites were detectable. Superscripts indicate the number of groups in an experiment. Butylated hydroxyanisole was estimated chromatographically, and glucuronides chromatographically after enzymic hydrolysis, both as isomer B. Ethereal sulphates were estimated gravimetrically.

Expt. no.	Dose (mg./rat)	Dose (g./kg.)	Butylated hydroxyanisole in medium (%)	Free butylated hydroxyanisole	Period of excretion (days)	Glucuronide	Period of excretion (days)	Ethereal sulphate	Period of excretion (days)	Total accounted for
8[1]	31·5	0·1	10	4	7–8	75	5–6	12	3–4	91
9[1]	13·5	0·05	10	5	3–4	69	5–6	10	2–3	84
10[1]	7·6	0·025	1	8	3–4	92	4–5	Not detectable	.	100
11[1]	3·7	0·01	1	14	4–5	.	.	Not detectable	.	.
12[2]	3·6, 3·9	0·01	1	7, 12	2–3	70, 74	2–3	Not detectable	.	81, 82
13[3]	0·82, 0·85	0·002	0·35	9·5, 11	2–3	72*	2–3	Not detectable	.	82

* Estimated on the pooled daily output of both groups.

Table 5. *Urinary excretion of butylated hydroxyanisole and metabolites during successive doses of* 0·5 *g./kg.*

Results are expressed as % of dose fed. Compounds were injected by gastric tube: (*a*) as a 50% (w/v) solution in propylene glycol; (*b*) as a 10% (w/v) suspension in diluted pea soup; (*c*) as a 50% (w/v) solution in corn oil. Each rat of a group of rats was given successive daily doses. Urines were collected for the duration of dosing and pooled. Unless specified otherwise, sulphates were measured gravimetrically and glucuronides by the modified naphtharesorcinol method. Superscript numerals indicate the number of doses per rat.

Compound	Expt. no.	Total average dose/rat (g.)	Medium	No. of rats	Free butylated hydroxy-anisole	Gluc-uronide	Ethereal sulphate	Total accounted for
	14	0·81	*b*	4^5	6	47	23*	76
Isomer A	15	0·77	*c*	6^5	5 1‡	24† 10‡	30 5‡	59 16‡
	16	0·52	*a*	3^5	7	43	6*	56
Isomer B	17	1·05	*b*	6^4	2	70†	3	75
Butylated hydroxyanisole	18	1·94	*b*	8^{10}	3	46	3	52

* Estimated by Sperber's method (Bray & Thorpe, 1954). † Estimated enzymically.
‡ Excreted in 12 days after the last dose in Expt. 15.

Glucuronide gum (pH 9·5). The aqueous solution (750 ml.), mixed with saturated barium acetate solution (10 ml.) and adjusted to pH 10·0, was slowly concentrated *in vacuo*. Needles of the barium salt of the glucuronide of isomer B (10·2 g.) separated and were converted into 2-*tert.*-butyl-4-methoxyphenyl-β-glucosiduronic acid, m.p. 168–169°, by the method of Dacre *et al.* (1956) (Found: C, 57·5; H, 6·8; Calc. for $C_{17}H_{24}O_8$: C, 57·3; H, 6·8%). The i.r. spectrum had a strong band at 5·75 μ (carboxyl C:O) and enhanced absorption, compared with isomer B, at 3 μ (OH).

The supernatant of the glucuronide precipitation was freed from Ba^{2+} ions with CO_2, and the filtrate gave on evaporation *in vacuo* a clear-yellow gum (19·4 g.), shown by enzymic hydrolysis to contain 0·39 g. of unprecipitated glucuronides of butylated hydroxyanisole. The gum was twice extracted with hot ethanol (100 ml.) and since the ethanol-insoluble portion contained no metabolites it was discarded. Comparison of the ethanol-soluble gum with the corresponding gum from control rat urine by enzymic hydrolysis and two-dimensional chromatography of the ether extract of the hydrolysate revealed that the only glucuronides not normally present were those of isomer A and B.

Residual urine. After the removal of lead with H_2S, the combined filtrate and washings (1·5 l.) were concentrated *in vacuo* to low volume, stirred for 1 hr. with stearic acid-deactivated charcoal (15 g.; Asatoor & Dalgliesh, 1956), the charcoal was collected and the filtrate, which was chromatographically free from detectable metabolites of butylated hydroxyanisole, discarded. The substances adsorbed by the washed charcoal were removed by elution with 1 l. each of (successively) aq. 7·2% (v/v) phenol, aq. 6N-NH_3 soln.–aq. 7·2% (v/v) phenol (1:5, v/v) and 6N-HCl–aq. 7·2% (v/v) phenol (1:5, v/v). Paper-chromatographic examination of the phenol-free residues from the eluates revealed no substances, other than a trace of compound H, which were not present in the corresponding fractions of control urine.

Metabolism of isomer A. (i) *Isolation of the glucuronide as its amide.* Clarified urine (260 ml.) from Expt. 14 was shown chromatographically to contain compound H and isomer A; its ether extract contained isomer A, the impurity *p*-methoxyphenol and normally occurring phenols. The pH 4·5 glucuronide gum yielded no combined isomer A, and enzymic hydrolysis showed the pH 9·5 glucuronide gum (1·9 g.) to contain the glucuronides of isomer A and, in trace amounts, of *p*-methoxyphenol. Solvent extraction of this gum, partition chromatography, methylation and acetylation yielded intractable products. It was extracted with cold propan-2-ol (3 × 350 ml.), the slightly dextrorotatory extract was evaporated to an amorphous solid which was methylated and acetylated, the dried product extracted with boiling hexane (200 ml.) and the extract chromatographed on a column of acid-washed alumina prepared from a hexane slurry. Fractions (100 ml.) were selected by extinction at 280 mμ; those eluted in order by benzene–$CHCl_3$ (2:3, v/v), benzene–$CHCl_3$ (1:4, v/v) and $CHCl_3$ were combined and evaporated. The residue was dissolved in methanol (20 ml.), the chilled solution was saturated with anhydrous NH_3 and kept for 24 hr. at 5°. The methanol was removed and the residue crystallized from water to yield white prisms of 3-tert.-*butyl*-4-*methoxyphenyl*-β-*glucosiduronamide* (8·5 mg.), m.p. 237·0–238·5° (decomp.) not depressed on admixture with a synthetic specimen (Found: C, 57·1; H, 7·5. $C_{17}H_{25}O_7N$ requires C, 57·4; H, 7·1%); natural and synthetic materials had identical u.v. and i.r. absorptions. We were unable to obtain the barium salt of this glucuronide prepared by Dacre *et al.* (1956).

(ii) *Isolation of the ethereal sulphate.* The copious precipitate of lead salts, obtained at pH 9·5 from clarified urine (510 ml.) from Expt. 15, was suspended in water and lead was removed as PbS; the solution was partly concentrated at 35° *in vacuo* to remove H_2S, adjusted to pH 9·5 with K_2CO_3 and evaporated *in vacuo* at 40° to a gum. This was extracted with cold ethanol (500 ml.), and the ethanol extract yielded a clear gum, from which, on addition of saturated Safranine bluish solution (10 ml.), red needles separated (1·26 g.), which crystallized from aq. ethanol. 3-*Amino*-7-*dimethylamino*-5-*phenylphenazinium* 3-tert.-*butyl*-4-*methoxyphenyl sulphate* (0·42 g.) had m.p. 230–235° (Found: C, 64·4; H, 5·9; N, 10·4. $C_{31}H_{34}O_5N_4S$ requires C, 64·8; H, 6·0; N, 9·7%); i.r. and u.v. spectra were identical with those of synthetic material (m.p. 233–235°), also R_F (0·87) in solvent I. The Safranine salt (400 mg.) was shaken for 2 hr. with Dowex-50 (K^+ form), and the solution was filtered and concentrated *in vacuo*; the residue, recrystal-

lized from ethanol, yielded *potassium* 3-tert.-*butyl*-4-*methoxyphenyl sulphate* (74 mg., 37%). The plates, recrystallized from propan-2-ol, had an indeterminate m.p. (Found: C, 43·8; H, 5·2; K, 13·1. $C_{11}H_{15}O_5SK$ requires C, 44·3; H, 5·1; K, 13·1%); i.r. and u.v. spectra, chemical and chromatographic properties were identical with those of synthetic material.

Properties and attempted isolation of compound H. Compound H was detected on chromatograms in solvent I of urine from all rats dosed with isomer A. Its absence from urine of rats dosed with *p*-methoxyphenol showed that it was not a metabolite of this impurity in isomer A. Compound H remained in urine after extraction with ether and after precipitation of glucuronides. Lead-free urine thus treated from Expt. 15 was concentrated to a stiff gum which was macerated under hot acetone (1 l.) and the extract evaporated to a gum; the acetone-insoluble portion was discarded. The gum was stirred in water (100 ml.) for 1 hr. with stearic acid-deactivated charcoal, when compound H was completely adsorbed. The adsorbed substances were eluted with aq. 7·2% (v/v) phenol and aq. 6N-NH_3 soln.–aq. 7·2% (v/v) phenol (1:5, v/v), and the eluates on evaporation *in vacuo* and removal of phenol yielded gummy residues which were combined (0·52 g.), streaked on eight sheets of Whatman no. 4 paper, 18·5 in. × 22 in., and chromatographed in solvent I. Compound H, located by co-chromatography, was eluted from the required cut-out zones with solvent I and the eluate was evaporated to a brown gum, which was washed with benzene and propan-2-ol. The light-brown semi-crystalline residue (10 mg.) had the reactions shown in Table 1 and the light-absorption maxima shown in Table 2. Compound H gave no Fe^{3+} ion or ninhydrin reactions on paper and no naphtharesorcinol reaction in solution. The i.r. spectrum had bands at 3·0 and 9·5 μ (S:O). Gentle warming with 3N-HCl liberated SO_4^{2-} ions and an ether-extractable phenol, which had the chromatographic properties (Table 1) and the u.v. spectrum of *tert.*-butylquinol. Both the liberated phenol and *tert.*-butylquinol gave in aq. ethanol the same pale-yellow Fe^{3+} ion colour, which deepened on addition of 1 drop of 2N-NaOH. Compound H is probably a 2 (or 3)-*tert.*-butyl-4-hydroxyphenyl sulphate, derived from isomer A by *O*-demethylation and subsequent ethereal sulphate conjugation of either hydroxyl, or by *O*-demethylation of the ethereal sulphate of isomer A.

Metabolism of isomer B. Continuous ether extraction of the urine (120 ml.) from Expt. 16 yielded isomer B, b.p. 150–155° at 35 mm. Hg, identical with the material fed. The glucuronide gum yielded 2-*tert.*-butyl-4-methoxyphenyl-β-glucosiduronic acid (0·36 g.) as before, m.p. and mixed m.p. 168–169°. Exhaustive chromatographic examination of the enzymic hydrolysates of the glucuronide gum and of the glucuronide-free urine revealed no further metabolites of isomer B. No ethereal sulphate Safranine bluish salt was obtained from the glucuronide gum.

Synthesis of ethereal sulphates

These were prepared by the method of Feigenbaum & Neuberg (1941).

Potassium 3-tert.-*butyl*-4-*methoxyphenyl sulphate.* A mixture of carbon disulphide (20 ml.), diethylaniline (5 ml.) and chlorosulphonic acid (2 g.) was slowly added with stirring to a chilled solution of isomer A (3 g.) in carbon disulphide (20 ml.) and diethylaniline (2 ml.). After 3 hr., an excess of aq. 50% (w/v) KOH was added slowly, followed by ether (200 ml.); the precipitate was triturated with ether (200 ml.) and extracted with hot propan-2-ol. The cooled extract yielded colourless prisms (2·4 g., 48%) of indeterminate m.p. (Found: C, 44·2; H, 5·0; S, 10·8; OCH_3, 11·0; K, 13·1. $C_{11}H_{15}O_5SK$ requires S, 10·7; OCH_3, 10·4%) having absorption at 9·42 μ (S:O). It decomposed at 65° *in vacuo* and above 120°. On gentle warming of a portion with 2N-HCl, isomer A was detected chromatographically in the ether extract of the solution and the solution also gave a strong SO_4^{2-} ion reaction. An insoluble lead salt was formed at pH 9·5. Addition of saturated aq. Safranine bluish solution to aq. 10% (w/v) potassium 3-*tert.*-butyl-4-methoxyphenyl sulphate yielded lustrous green–red needles, recrystallized from aq. ethanol. 3-*Amino*-7-*dimethylamino*-5-*phenylphenazinium* 3-tert.-*butyl*-4-*methoxyphenyl sulphate* had m.p. 233–235° (Found: C, 64·8, 64·7; H, 5·8; 6·0; N, 9·9; S, 5·3. $C_{31}H_{34}O_5N_4S$ requires S, 5·6%), R_F in solvent I, 0·87. It had $\lambda_{max.}$ 229 (log $\epsilon_{max.}$ 4·28) and 274 mμ (log ϵ, 4·69). The Safranine salt (50 mg.) in water (10 ml.) was shaken for 1 hr. with Dowex-50 (K^+ form) (5 g.), the solution was filtered, evaporated to dryness *in vacuo* and the residue, recrystallized from propan-2-ol, yielded potassium 3-*tert.*-butyl-4-methoxyphenyl sulphate (16·4 mg., 65%).

Potassium 2-tert.-*butyl*-4-*methoxyphenyl sulphate.* This, prepared as described above from isomer B in 50% yield, recrystallized from propan-2-ol as prisms, had an indeterminate m.p. (Found: C, 44·3; H, 5·2; S, 10·7; OCH_3, 10·1; K, 13·2. $C_{11}H_{15}O_5SK$ requires C, 44·3; H, 5·1; S, 10·7; OCH_3 10·4; K, 13·1%) and strong absorption at 9·45 μ. Its chemical properties were similar to those of the ethereal sulphate of isomer A. 3-*Amino*-7-*dimethylamino*-5-*phenylphenazinium* 2-tert.-*butyl*-4-*methoxyphenyl sulphate*, obtained as before, gave red needles from aq. ethanol, m.p. 216–218° (Found: C, 63·8; H, 6·1. $C_{31}H_{34}O_5N_4S$, 0·5 H_2O requires C, 63·8; H, 6·0%) and had $\lambda_{max.}$ 229 (log $\epsilon_{max.}$ 4·28) and 273 mμ (log $\epsilon_{max.}$ 4·69).

Synthesis of derivatives of the glucuronide of isomer A

Methyl (*tri*-O-*acetyl*-3-tert.-*butyl*-4-*methoxyphenyl*-β-*glucosid*)*uronate.* This was prepared by the procedure of Bollenback, Long, Benjamin & Lindquist (1955). Methyl-(tetra-*O*-acetyl-β-glucopyranosid)uronate (5 g.), isomer A (5 g.) and freshly fused $ZnCl_2$ (1 g.), dissolved in 20 ml. of acetic anhydride–acetic acid (1:20, v/v), were heated at 100° for 20 min., excess of solvent was removed *in vacuo* at 100° for 20 min., the residue was dissolved in benzene (100 ml.) and the solution washed with 2N-NaOH (2 × 100 ml.) and water (100 ml.), dried over $MgSO_4$ and concentrated *in vacuo*. A semi-crystalline residue resulted after several hours at 1·5 mm. Hg, and after trituration with hexane colourless needles were collected and recrystallized from hexane. *Methyl* (*tri*-O-*acetyl* 3-tert.-*butyl*-4-*methoxyphenyl*-β-*glucosid*)*uronate* (1·6 g.) had m.p. 122–123°, $[\alpha]_D^{24}$ – 25·1° in $CHCl_3$ (*c*, 16·7) (Found: C, 57·6; H, 6·7; OCH_3, 12·9. $C_{24}H_{32}O_{11}$ requires C, 58·0; H, 6·5; OCH_3, 12·5%). Chromatographic treatment of the combined mother liquors on a column of acid-washed alumina yielded a further 600 mg. of product, m.p. 118–120·5° (total yield 15%). Longer heating of the reactants (90 min. at 100°) yielded, on chromatography of the product, needles of 3-tert.-*butyl*-4-*methoxyphenyl acetate* (4·5 g.),

m.p. 56–58°, identical with that prepared by acetylation of isomer A in pyridine and acetic anhydride, m.p. 58·0–58·5° (Found: C, 70·2; H, 8·5; OCH_3, 13·3. $C_{13}H_{18}O_3$ requires C, 70·2; H, 8·2; OCH_3, 13·9 %) and yellow needles (0·65 g.) of the anomeric *methyl (tri-O-acetyl-3-tert.-butyl-4-methoxyphenyl-α-glucosid)uronate*, m.p. 144–146°, $[\alpha]_D^{24}$ +127° in $CHCl_3$ (*c*, 9·1) (Found: C, 58·4; H, 6·5 %), but no β-anomer. Anhydrous NH_3 was passed for 0·5 hr. into a chilled solution in methanol (30 ml.) of the β-anomer (300 mg.), the solution was chilled for 3 days, the solvent removed and the residue crystallized from water to yield needles (135 mg.) of *3-tert.-butyl-4-methoxyphenyl-β-glucosiduronamide*, m.p. 238·5° (decomp.), $[\alpha]_D^{24}$ – 188° in ethanol (*c*, 0·9) (Found: C, 57·0; H, 7·3; N, 4·3. $C_{17}H_{25}O_7N$ requires N, 3·9 %). The i.r. spectrum showed strong bands at 2·84, 2·93 and 5·98 μ and the u.v. spectrum had $\lambda_{max.}$ 226 (log ε 3·93) and 284 mμ (log ε, 3·43).

Attempted preparation of the glucuronide of isomer A. Hydrolysis of the methyl tri-*O*-acetyl-β-anomer with $Ba(OH)_2$ solution or aq. methanolic suspensions of $Ba(OH)_2$ yielded amorphous products. Catalytic deacetylation of 1·34 g. with sodium (0·05 g.) in methanol (300 ml.) yielded, after 6 days, a semi-crystalline mass on evaporation *in vacuo*, which was purified through its lead salt or over Dowex-2 (H^+ form) to give a residue which separated from propan-2-ol as a glass (270 mg.), $[\alpha]_D^{24}$ – 46·5° in water (*c*, 3·4). The product gave a strong positive naphtharesorcinol reaction and had pH 3·7 in 0·1 % solution. Hydrolysis with β-glucuronidase (10^4 Fishman units) of 2·8 μmoles gave 2·45 μmoles (84 %) of isomer A, estimated chromatographically, and u.v. (Table 2) and i.r. spectra (bands at 3·0, 5·8 μ) were closely similar to those of the glucuronide of isomer B. This was the purest specimen prepared of the glucuronide of isomer A.

DISCUSSION

The metabolism of butylated hydroxyanisole in the rat resembles that reported for the rabbit for doses of 0·13–0·55 g./kg. (Dacre *et al.* 1956). At all doses studied in the rat butylated hydroxyanisole and its component isomers, on ingestion, are absorbed and readily metabolized. The possible metabolic pathways for phenols and substances analogous to butylated hydroxyanisole (Williams, 1949; Brodie, Gillette & LaDu, 1958) suggest that 4-*O*-conjugation, *O*-demethylation, hydroxylation of the phenyl ring and side-chain oxidation ($-CH_3 \rightarrow CH_2{\cdot}OH$) might be involved in metabolism of butylated hydroxyanisole. However, the main metabolites are 4-*O*-conjugates, i.e. the *O*-sulphate and *O*-glucuronide of isomer A and the *O*-glucuronide of isomer *B*. The only other metabolic pathway is the probable *O*-demethylation of isomer A, which yields compound H. In the rabbit both isomers are metabolized largely by 4-*O*-glucuronide formation (Dacre *et al.* 1956).

The conjugation of isomer B appears to differ from that predictable from steric considerations. The 4-hydroxyl group in isomer B is clearly crowded by the 3-*tert.*-butyl group, such that conjugation with the ethereal sulphate group would seem preferable to conjugation with the bulkier glucuronide group (Fisher–Taylor–Hirschfelder Models, Fisher Scientific Co., Pittsburgh, Pa., U.S.A.). However, isomer B forms mostly an *O*-glucuronide. Isomer A does not appear to possess steric restrictions on conjugation, and forms mainly an ethereal sulphate, *O*-glucuronide formation apparently becoming significant only when the sulphate pool is depleted by repeated large doses.

In the single-dose studies, at the lowest dose (2 mg. of butylated hydroxyanisole/kg.) the background absorption of chromatograms in the zones examined for isomer B, owing to the excretion of normal metabolites, is no longer negligible. Thus the apparent glucuronide excretion (enzymic method) of controls, expressed as isomer B, amounts to 18 % (0–39 %)/day of a dose of 0·82 mg. of butylated hydroxyanisole/rat. The apparent excretion of free isomer B by controls amounts to 6 % (0–13·5 %)/day of the same dose. In Expt. 13, however, actual control values, determined on the day before dosing, were glucuronide, 0 %, and free isomer B, 3·8–4·0 %, of the dose, and were assumed to be unaltered during urine collection. In other experiments at this dose, not reported, background absorption was too high to enable a reliable estimation of dose excretion to be made. This dose is clearly a lower limit for the method.

Over the range studied, the excretion of unconjugated butylated hydroxyanisole decreased from 11 % (7–14 %) for a 10 mg./kg. dose to 3–7 % for a 400 mg./kg. dose. With 0·3 mg./kg. in dogs H. C. Hodge & D. W. Fassett (unpublished) found negligible tissue storage, and in the present study over-all recoveries of a single dose remained uniformly high (82–100 %) at all doses studied. The slightly higher proportion of unconjugated butylated hydroxyanisole excreted at the lowest doses is therefore unlikely to be associated with any increase in tissue storage.

The above study approaches closer to human-usage doses than does any previous study on antioxidants, and, although the problem of studying the human-usage dose remains, extrapolation to that amount seems justified. No evidence has so far been presented which suggests that butylated hydroxyanisole is not largely absorbed and largely excreted at these very low doses.

SUMMARY

1. The metabolism of butylated hydroxyanisole and 2- and 3-*tert.*-butyl-4-hydroxyanisole in the rat is studied with particular emphasis on the fate of butylated hydroxyanisole at low doses.

2. Increased urinary glucuronide and ethereal sulphate excretions during 5 days after dosing with

0·4 g. of butylated hydroxyanisole/kg. account for 61–82 and 11–16% respectively of the dose, and 5% of the dose is excreted, unchanged.

3. For single oral doses of 0·002, 0·01, 0·025, 0·05 and 0·1 g. of butylated hydroxyanisole/kg., overall recoveries of the dose from the urine were 81–100%. Glucuronide excretion accounts for 69–92% of the dose, and a slight increase in excretion of unchanged butylated hydroxyanisole occurs at the lowest doses.

4. For single oral doses of 0·4 g. of 2-*tert.*-butyl-4-hydroxyanisole 72% of the dose is excreted as ethereal sulphate in 5 days. With 3-*tert.*-butyl-4-hydroxyanisole 57–71% of the dose is excreted as glucuronide in 5 days.

5. The administration of several successive daily doses of 0·5 g. of butylated hydroxyanisole/kg., and of 3-*tert.*-butyl-4-hydroxyanisole, decreases the percentage recovery of conjugates and the proportion of ethereal sulphate excreted during dosing; repeated doses of 0·5 g. of 2-*tert.*-butyl-4-hydroxyanisole/kg. cause a considerable increase in glucuronide and a decrease in ethereal sulphate conjugation of the dose.

6. The isolation of the major metabolites, the probable formation of a demethylation product of 2-*tert.*-butyl-4-hydroxyanisole as a minor metabolite, the synthesis of the ethereal sulphates of both isomers and the synthesis of the glucuronamide of 2-*tert.*-butyl-4-hydroxyanisole are described.

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Partial Purification and some Properties of the Blood-Clotting Factor from the Venom of *Bothrops jararaca*

By OLGA B. HENRIQUES, MINA FICHMAN and S. B. HENRIQUES
Instituto Butantan, C.P. 65, São Paulo, Brazil

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The blood-clotting activity of the venom of various species of *Bothrops* snakes has been studied by various authors, some of them attributing the clotting effect to the proteolytic activity of the venom (Jánszky, 1950; Zeller, 1951; Slotta, 1953), and others (Taborda, 1940; Deutsch & Diniz, 1955; Rosenfeld, Hampe & Kelen, 1960) giving evidence that separate enzymes are concerned with the two activities. Michl (1954) and Holtz & Raudonat (1956) were able to show that in the venom of *Bothrops jararaca* the clotting activity is distinguishable from its proteolytic activity. However, Hamberg & Rocha e Silva (1956) and Henriques, Lavras & Fichman (1956) have shown that in the venom of *B. jararaca* there exist at least two proteolytic enzymes. Therefore one of them could be responsible for the clotting activity. Nevertheless, Henriques, Mandelbaum & Henriques (1959) demonstrated that the clotting activity of the venom of *Bothrops jararaca* is independent of those

two proteolytic enzymes, namely 'caseinase' (Henriques *et al.* 1956) and 'protease' (Holtz & Raudonat, 1956), and *Bothrops* protease A, which hydrolyses benzoyl-L-arginine amide but not casein (Henriques, Lavras, Fichman, Mandelbaum & Henriques, 1958). Habermann (1958), by fractional precipitation with acetone followed by paper electrophoresis, obtained from the venom of *Bothrops jararaca* a fraction that had a 'relative activity' ten times as high as that of the starting material with respect to blood-clotting activity.

In this paper is given a detailed account and extension of observations previously made by Henriques *et al.* (1959). It contains also the study of some properties of the clotting factor and the results of an attempt to isolate it.

MATERIALS AND METHODS

Clotting activity. The clotting activity was measured by adding 0·2 ml. of oxalated horse plasma (0·3 g. of potassium oxalate/100 ml. of plasma) to 0·1 ml. of the sample previously placed on a water bath at 35°; the zero time was noted and the mixture gently agitated until a firm clot was formed. The samples containing the clotting factor were diluted twice or more with 0·2M-sodium cacodylate buffer, pH 6·5. This concentration of buffer was used to obtain pH 7·0 in the final reaction mixture of sample and plasma.

Specific clotting activity. Amounts of venom which clot blood plasma or fibrinogen at between 20 and 50 sec. show a straight-line relationship when the reciprocals of enzyme concentration are plotted against the respective clotting times. Therefore when testing a sample for its blood-clotting activity, three different concentrations of protein were used and, by graphical interpolation, the inverse of the protein concentration which would cause clotting in 30 sec. was inferred from the straight line obtained. This figure, multiplied by 30 for convenience, was taken as the specific clotting activity of the sample analysed, and proved to be accurate enough for the purposes of the present work.

Proteolytic activities. The activities of the samples towards casein and benzoyl-L-arginine amide were determined respectively by the methods of Kunitz (1946) and Schwert, Neurath, Kaufman & Snoke (1948) as previously described (Henriques *et al.* 1958).

Fractionation of the venom. The venom used for this work was collected about 1 month before starting the experiments here reported, the samples being immediately dried *in vacuo* over $CaCl_2$ at room temperature. The whole procedure of fractionation and subsequent dialysis was done at 5–10°. The venom (2 g.) was dissolved in 100 ml. of 0·05M-sodium cacodylate buffer, pH 6·10, and centrifuged to remove the insoluble residue. The proteins of the venom were fractionally precipitated with $(NH_4)_2SO_4$. The solid salt, divided into small portions, was stirred with the venom solution to give the desired percentage saturation; the amount of $(NH_4)_2SO_4$ added was calculated from the table of Green & Hughes (1955). The precipitates were centrifuged, dissolved in a small volume of 0·05M-sodium cacodylate buffer, pH 6·10, and dialysed in cellophan tubing against the same buffer until the diffusate was free of NH_4^+ ions.

Protein determination. Protein was estimated photometrically by the biuret method of Wolfson, Cohn, Calvary & Ichiba (1948) or in terms of its ultraviolet absorption at 280 mμ in the 1 cm. cell of a Beckman spectrophotometer.

Electrophoresis on starch columns. The procedure was the same as that described by Henriques *et al.* (1958).

Activators and inhibitors. Solutions of activators or inhibitors were always freshly prepared in water and incubated for 1 hr. at 35° with the enzyme before the addition of the oxalated plasma.

RESULTS

It was observed that the clotting activity varies considerably with the buffer employed. Fig. 1 shows the clotting activity in the presence of cacodylate, phosphate and acetate buffers. In the presence of 0·2M-sodium cacodylate the activity increased with the pH, reaching a plateau at pH 6·5 which was maintained throughout the upper useful range of the buffer. In the presence of 0·2M-phosphate (NaH_2PO_4–Na_2HPO_4) buffer, pH 6·2, the activity was comparable with that shown with cacodylate buffer. With the rise in pH, however, the activity fell sharply. In the presence of 0·2M-acetate (sodium acetate–acetic acid) buffer the clotting activity was shown to be higher than in the presence of cacodylate below pH 5·6, the situation being reversed at higher pH values. Other buffers, such as citrate, 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–hydrochloric acid and tris maleate were tried at the pH of maximal activity in cacodylate. In all cases the activity was lower than in the presence of cacodylate. Cacodylate buffer has the further advantage of main-

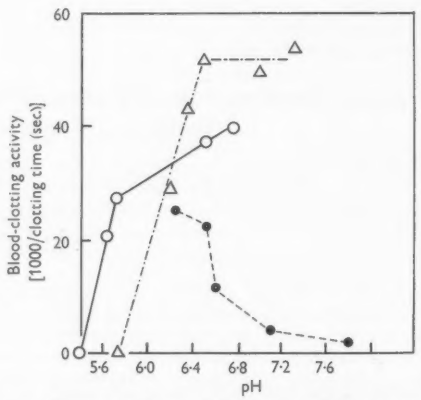

Fig. 1. Effect of pH and buffers on the blood-clotting activity of the venom of *B. jararaca*. Reaction mixture contained 0·1 ml. of 0·1% (w/v) solution of venom in the corresponding 0·2M-buffer and 0·2 ml. of oxalated horse plasma. ○, Acetate; ●, phosphate; △, cacodylate.

taining unaltered, for at least 2 months, the clotting activities both of the solution of venom and of its fractions, when kept at 1–2° at pH 6·2.

Effect of activators and inhibitors. In these experiments the effect of the ions was tested in a crude venom solution and in a fraction purified ninefold by $(NH_4)_2SO_4$ fractionation and electrophoresis on a starch column. It can be seen from Table 1 that the ions tested affected both samples in the same way. In the presence of 2 mM-metal-ion concentration only Cu^{2+} and Hg^{2+} ions had an inhibitory effect (Table 1). Calcium had a slight activating effect, confirming a previous report by Hargreaves (1944). In the presence of 1 mM-iodoacetate, -fluoride, -sulphide, -cyanide and -azide, no effect was observed. Reduced glutathione in 50 mM concentration and ethylenediaminetetra-acetic acid (disodium salt) in 2 mM concentration strongly inhibited the clotting activity (Table 2). The inhibition by ethylenediaminetetra-acetic acid has been previously reported by Rosenfeld & Jánszky (1952).

Effect of heating. As shown in Table 3, heating the venom to 63° in 0·05 M-sodium cacodylate buffer, pH 6·2, followed by immediate cooling in an ice bath, reduces the clotting specific activity to 25 % of that of the crude venom, and heating to 87° followed by cooling in the same conditions reduces it to about 12 %. By contrast, the amidase specific activity, confirming previous observations (Hamberg & Rocha e Silva, 1956, 1957; Henriques *et al.* 1958), is more heat resistant, its activity being reduced to 75 % at 63° and to 60 % at 87°. The caseinase specific activity falls to 50 % at 63° and is completely destroyed when the venom is heated to 83°.

Separation of the clotting fraction from the venom. It can be observed from Table 4 that the highest clotting activity was found in the fraction precipitated with $(NH_4)_2SO_4$ between 0·60 and 0·65 saturation. This confirms a previous report (Henriques *et al.* 1959), in which the highest clotting activity was observed at 0·60–0·70 saturation with $(NH_4)_2SO_4$. The most active fraction obtained in this experiment had a specific clotting activity 2·4 times as great (602) as that of the crude venom (Table 4, fraction 7). Table 4 shows that some

Table 1. *Effect of metal ions on the clotting activity of the venom of* Bothrops jararaca

Venom solution was incubated for 1 hr. at 35° with test substances (final concn. 2 mM) before addition of oxalated plasma. Reaction mixture for the determination of clotting activity: 0·1 ml. of venom or fraction and metal-binding-agent mixture and 0·2 ml. of oxalated horse plasma.

Metal ion	Clotting time (sec.) Crude venom	Purified fraction*
None	20·0	20·0
Mn^{2+}	19·5	19·0
Cu^{2+}	41·0	44·0
Ca^{2+}	16·0	14·5
Fe^{3+}	19·8	18·5
Ag^{+}	21·0	20·0
Pb^{2+}	19·5	19·0
Cd^{2+}	19·2	19·2
Co^{2+}	22·5	21·5
Zn^{2+}	19·5	17·5
Hg^{2+}	39·5	35·5
Mg^{2+}	19·5	17·8

* Similar to fraction 5, Table 5, obtained from venom collected in 1946; nine times purified with respect to clotting activity.

Table 2. *Effect of certain anions and metal-binding agents on the clotting activity of the venom of* Bothrops jararaca

Test substances were incubated for 1 hr. with venom before addition of oxalated plasma. Venom–test-substance mixture was incubated with plasma as described in Table 1.

Added substance	Final concn. (mM)	Clotting time (sec.)
None	—	20
Iodoacetate	1	21
Fluoride	1	20
Sulphide	1	21
Cyanide	1	21
Azide	1	21
Reduced glutathione	50	80
Ethylenediaminetetra-acetic acid (disodium salt)	2	240*

* Partially clotted.

Table 3. *Effect of heating on the clotting, benzoyl-L-arginine-amidase and caseinase specific activities of the venom*

A 2 % (w/v) solution of *B. jararaca* venom in 0·05 M-sodium cacodylate buffer, pH 6·2, was heated in a boiling-water bath until the desired temperature was reached. Immediately afterwards the tube containing the sample was transferred to an ice–water bath. Specific clotting activity is expressed as 30 (mg. of protein/ml. of the sample that clots the plasma in 30 sec.). Benzoylarginine-amidase specific activity is expressed as μg. of NH_3 liberated from benzoyl-L-arginine amide/mg. of protein in the sample. Caseinase specific activity is expressed as the increase in extinction of the trichloroacetic acid filtrate at 275 mμ/mg. of protein in the sample. All the reactions were performed under standard conditions as described in the text.

Temp. reached	Specific activities: Clotting	Amidase	Caseinase
Room temp.	120	37	1·85
63°	30	28	0·96
87°	15	22	Nil

Table 4. *Blood-clotting activity and hydrolytic activity towards casein and benzoyl-L-arginine amide of fractions precipitated with ammonium sulphate*

Precipitation with $(NH_4)_2SO_4$ was done in 0·05M-sodium cacodylate buffer, pH 6·10. The clotting, amidase and caseinase specific activities are expressed as in Table 3. All the reactions were performed under standard conditions as described in the text. U indicates 'undetectable'.

Fraction no.	Saturation with $(NH_4)_2SO_4$	Specific activities			Yield (%)			
		Clotting	Amidase	Caseinase	Protein	Clotting	Amidase	Caseinase
1	None	248	32	2·05	100	100	100	100
2	0·35–0·40	77·5	10·6	2·30	5·5	1·7	1·8	12·8
3	0·40–0·45	162	17·8	2·00	10·3	6·7	5·7	21
4	0·45–0·50	219	27	2·50	19·0	16·8	16·0	47
5	0·50–0·55	318	32	1·60	18·4	23·6	18·6	29
6	0·55–0·60	511	51	0·86	12·2	25·2	19·6	10·0
7	0·60–0·65	602	71	0·31	7·8	19·0	17·4	4·9
8	0·65–0·70	456	98	0·17	2·7	0·50	8·3	0·46
9	0·70–0·80	U	154	0·20	2·3	—	1·1	0·54
10	0·80–1·0	U	22·6	0·18	0·23	—	0·17	0·042

Table 5. *Comparison of the clotting, caseinase and benzoylarginine-amidase specific activities of samples obtained during purification of the clotting activity of a mixture of fractions* 6, 7 *and* 8 *of Table* 4

The specific activities are expressed as described in Table 3. All the reactions were performed under standard conditions. U indicates 'undetectable'.

Fraction no.	Step	Specific activities			Yield (%)		
		Clotting	Amidase	Caseinase	Clotting	Amidase	Caseinase
1	Mixture of fractions 6, 7 and 8 (Table 4)	501	61	0·70	100	100	100
2	Reprecipitation of fraction no. 1 at 0–0·55 saturation with $(NH_4)_2SO_4$	517	47·5	0·93	56	42·6	75·9
3	Reprecipitation of fraction no. 1 at 0·55–0·60 saturation with $(NH_4)_2SO_4$	609	65	0·36	24·7	20·8	10·5
4	Reprecipitation of fraction no. 1 at 0·60–0·65 saturation with $(NH_4)_2SO_4$	570	94	0·19	6	8·2	1·5
5	Electrophoresis peak of clotting activity (starch column) of fraction no. 3	1020	127	U	2·98	1·4	—

clotting activity was found in all fractions, no sharp separation being obtained. In order to increase the yield, fractions 6, 7 and 8 (Table 4), which had similar specific clotting activities, were mixed and fractionally reprecipitated with $(NH_4)_2SO_4$. The highest specific clotting activity was obtained at a slightly lower saturation with $(NH_4)_2SO_4$, i.e. 0·55–0·60, and the specific activity reached the value 609 (Table 5), slightly higher than that of the initial mixture (501). A second reprecipitation with $(NH_4)_2SO_4$ did not lead to further purification. When this reprecipitated fraction was submitted to electrophoresis on a starch column (cacodylate buffer, pH 6·10, *I* 0·05) further purification was obtained, the clotting activity reaching the value 1020, i.e. about four times as great as that of the crude venom from which it was obtained. As can be seen from Fig. 2, a clear separation of the peaks of *Bothrops*-protease A and clotting activities was obtained.

It should be noted here that the degree of purification obtained depends on the age of the venom used. When the purified clotting factor was obtained from old venoms (1946–48) by the technique described in this paper, the overall increase in specific activity was about nine- to ten-fold instead of fourfold as given here. The final specific activity of the purified material obtained either from old or new venoms was, however, about the same.

Attempts were made to purify the clotting factor with gels of aluminum hydroxide (Colowick, 1955), calcium phosphate (Keilin & Hartree, 1938), barium carbonate (Fantl & Nance, 1947) and Amberlite IRC-50 (XE-64), varying buffers and pH. Doery (1958) reported the purification of the blood-

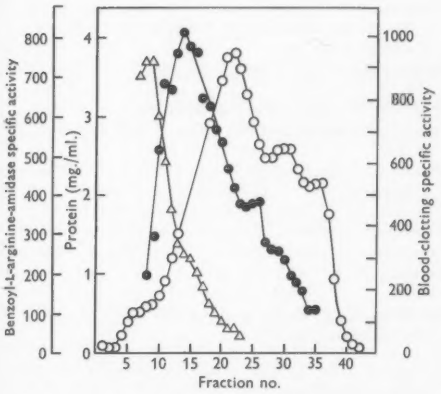

Fig. 2. Protein concentration and amidase and blood-clotting specific activities of fractions obtained by electrophoresis of 220 mg. of protein of fraction no. 3 (Table 5) on a starch column. Cacodylate buffer, pH 6·2, *I* 0·05; about 11 mA; under these conditions the proteins moved to the anode. △, Benzoyl-L-arginine-amidase specific activity; ●, blood-clotting specific activity; ○, mg. of protein/ml.

clotting factor of the venom of *Notechis scutatus scutatus* by adsorption on to barium carbonate. Treatment of the venom of *Bothrops jararaca* under the conditions specified by Doery (1958) did not lead to adsorption of the clotting enzyme on to barium carbonate. The clotting enzyme was not adsorbed by calcium phosphate but it was adsorbed by alumina-Cγ; however, the adsorption was not selective. On Amberlite IRC-50 (XE-64) the blood-clotting factor was adsorbed and eluted at pH 7·1 in cacodylate buffer when the tube test recommended by Hirs, Moore & Stein (1953) was used. However, when the ion-exchange resin was used in a column no purification was obtained.

DISCUSSION

The results presented in this paper give evidence that the blood-clotting factor differs from the two other proteolytic enzymes known to be present in the venom of *Bothrops jararacá*, namely *Bothrops* protease A and 'caseinase'. These proteolytic enzymes can already be distinguished from the blood-clotting enzyme by some of its properties. The caseinase is almost completely inhibited by Cd^{2+} ions (Henriques *et al.* 1958) whereas the clotting enzyme is not affected by the same concentration of Cd^{2+} ions. Heating the venom to 87° in cacodylate buffer (Table 3) decreased the benzoyl-arginine-amidase specific activity to about two-thirds, completely destroyed the caseinase activity and decreased the specific clotting activity to about one-tenth. The pH optimum found for the clotting enzyme was about 7·0 and the enzyme activity was found to be activated by cacodylate buffer and inhibited by tris–hydrochloric acid. The latter was found to be the best medium for caseinase and *Bothrops*-protease A activities and the pH optimum for both of these enzymes was found to be approx. 8·8 (Henriques *et al.* 1958). The three enzymes can be partially separated by ammonium sulphate precipitation (Table 4). The fractions precipitated at 0·50–0·55, 0·60–0·65 and 0·70–0·80 saturation were found respectively to contain the highest specific activity of caseinase, clotting enzyme and *Bothrops* protease A. By fractional reprecipitation of the most strongly clotting fractions a preparation was obtained which still contained a high specific activity as *Bothrops* protease A, but when it was submitted to electrophoresis on a starch column two distinguishable peaks of activity were obtained (Fig. 2), one for the blood-clotting enzyme and the other for *Bothrops* protease A. The partial separation of caseinase and the clotting enzyme by ammonium sulphate precipitation confirms previous work by Holtz & Raudonat (1956), who reported a fraction precipitated at 40 % saturation with ammonium sulphate, strongly proteolytic and with slight clotting activity and a fraction precipitated at 60 % which had strong clotting activity and was weakly proteolytic.

The clotting activity caused by *Bothrops* venom may be due to a third proteolytic enzyme. In fact evidence has been presented that Reptilase, a pharmaceutical preparation of clotting enzyme obtained from *Bothrops* venom, can split from fibrinogen a peptide (Blombäk & Vestermark, 1958) which in Dowex columns shows the same behaviour as peptide A, one of the two peptides which are liberated by thrombin from fibrinogen (Bettelheim & Bailey, 1952).

Data presented in this paper do not exclude the hypothesis proposed by Habermann (1959) that there exist in the venom three enzymes which hydrolyse N^{a}-toluene-*p*-sulphonyl-L-arginine methyl ester and, therefore, benzoylarginine amide. His hypothesis seems to be based on an electrophoresis diagram of purified blood-clotting enzyme, in which he plotted the yield of the N^{a}-toluene-*p*-sulphonyl-L-arginine methyl esterase activity against the effluent volume (Habermann, 1959; fig. 1*a*). As judged from the diagram published by Habermann the fraction he analysed was about as polydisperse as the one included in this paper (fig. 2). Under these circumstances it is questionable whether the appearance of three peaks of yield should be accepted as evidence for the existence of three distinct N^{a}-toluene-*p*-sulphonyl-

L-arginine methyl esterases in the venom; but a final conclusion must await further purification of the blood-clotting enzyme.

SUMMARY

1. The pH optimum for the blood-clotting activity of the venom of *Bothrops jararaca* was found to be about 7·0; at this pH the enzyme was more active in cacodylate buffer, being inhibited by phosphate, 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–hydrochloric acid, citrate and tris maleate. The clotting enzyme is thermolabile, its activity being reduced to about one-tenth when it is rapidly heated to 87°.

2. The venom fraction precipitated between 0·60 and 0·65 saturation with ammonium sulphate had a clotting activity about twice as high as that of the crude venom; its benzoylarginine-amidase specific activity was also about twice that of the crude venom; its caseinase specific activity was about one-sixth of that of the crude venom.

3. Fractional reprecipitation of the venom fraction precipitated between 0·60 and 0·65 saturation with ammonium sulphate followed by electrophoresis on a starch column produced a clotting fraction about four times as active as the crude venom.

4. The clotting enzyme was found to be different from the two other proteolytic enzymes found in the venom, namely 'caseinase' and *Bothrops* protease A.

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Carcinogenesis: Changes in the Properties of some Rat-Liver Proteins after Administration of 4-Dimethylamino-3′-methylazobenzene

By J. M. WHITCUTT, D. A. SUTTON and J. R. NUNN
National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, Pretoria, South Africa

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Many changes occur in rat-liver cells during the long period of induction between the first treatment with a carcinogenic azo dye and the appearance of actual cancer cells; these are described in an extensive literature which has been well reviewed. Histological differences are noticeable at an early stage (Chang, Spain & Griffin, 1958), and changes in the chemical composition of the cells have been reported (see Miller & Miller, 1953).

A significant development in recent years has been the attempt by Miller and co-workers to correlate carcinogenicity with the covalent binding of a small proportion of the carcinogen to certain liver proteins. It has been reported that: (i) appreciable binding of azo dye occurs only in the liver of the rat, i.e. the susceptible organ of the susceptible species (Miller & Miller, 1947; Salzberg, 1958); (ii) the rapidity with which maximum binding is reached is proportional to the potency of the carcinogen (Miller, Miller, Sapp & Weber, 1949); (iii) conditions which inhibit carcinogenesis also inhibit dye-binding (Miller & Miller, 1955; Gelboin, Miller & Miller, 1958). Azo carcinogens are not bound to the proteins of liver tumours (Miller & Miller, 1947), and the particular group of proteins to which they are bound in normal cells is largely absent from tumour cells (Sorof & Cohen, 1951). These results are in harmony with the theory that malignant change occurs through the gradual deletion of key proteins essential for growth control (Miller & Miller, 1953, 1955).

Recent work on azo-dye carcinogenesis has described the distribution of bound dye among the various cell components (Price, Miller, Miller & Weber, 1949; Hultin, 1955, 1957; Hughes, 1956; Salzberg, 1958; Hultin & Decken, 1958), the effect of diet upon dye-binding (Conney, Miller & Miller, 1956; Gelboin *et al.* 1958), distribution of dye in the soluble liver proteins (Sorof, Golder & Ott, 1954; Sorof, Young & Ott, 1958) and degradative examination of the proteins binding the dye (Wirtz & Arcos, 1958; Kusama & Terayama, 1957).

The present investigation is an attempt to study some of the changes occurring in the soluble liver proteins of rats a few hours after oral administration of the carcinogenic azo dye 4-dimethylamino-3′-methylazobenzene.

EXPERIMENTAL AND RESULTS

Ultraviolet spectra were measured on a Unicam model SP. 500.

Phosphate buffers were prepared by mixing stock solutions of disodium hydrogen phosphate (0·1 M) and potassium dihydrogen phosphate (0·1 M), and adding the calculated amount of sodium chloride and diluting. The borate buffer was prepared by diluting a stock solution containing boric acid (0·3 M) and sodium hydroxide (0·06 M) and adding the calculated amount of sodium chloride.

Protein solutions were stored at −20°; operations were carried out below 5° wherever possible, and were usually completed within 14 days of isolating the original material. Solutions of large volume were concentrated by standing overnight over calcium chloride at 2 mm. mercury pressure; those of small volume were concentrated and dialysed by means of the apparatus shown in Fig. 1.

Synthesis of 4-dimethylamino-3′-methylazobenzene. (Based on the synthesis of methyl red given in *Organic Syntheses*, Collective Volume 1.) *m*-Toluidine (20 ml.) in hydrochloric acid solution (150 ml. of 3·5 N) was diazotized by slowly adding a solution of sodium nitrite (15·2 g. in 36 ml. of water). After 1 hr. at 0° a solution of dimethylaniline (34 ml.) in dilute hydrochloric acid (75 ml. of water and enough conc. hydrochloric acid to dissolve) was added slowly with stirring. The mixture was allowed to stand for 2 hr. at 0° before adding a solution of sodium acetate (150 ml. of 4 M), and standing for a further 3 hr. with occasional stirring. The product was filtered off, recrystallized several times from ethanol and from benzene, and finally purified by chromatographing it on neutral alumina (E. Merck, Darmstadt, Germany) with *iso*hexane as solvent. M.p. 119·5–120·5° (Found: C, 75·5; H, 7·0; N, 17·1. Calc. for $C_{15}H_{17}N_3$: C, 75·28; H, 7·16; N, 17·56%).

Preparation of livers. The animals were provided by the National Nutrition Research Institute, Pretoria. Young male albino rats (wt. 200–250 g.) of the same strain were housed in wire cages at 20° and fed *ad lib.* on the Institute's stock diet (protein, 20%; ash, 7·4%; main component: maize meal, 56%) and water. A solution of 4-dimethylamino-3′-methylazobenzene (10 mg.) in olive oil (0·5 ml.) was administered orally in one dose and the animals were starved thereafter; in control experiments the same pro-

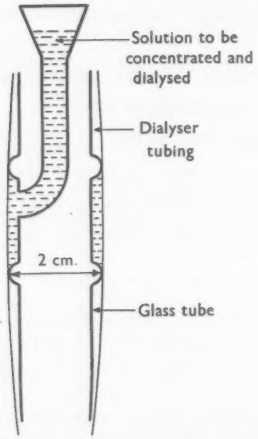

Fig. 1. Sectional view of apparatus for concentrating and dialysing small volumes of protein solutions. The dialyser tubing (visking) seals off the space between the ridges on the outside of the glass tube (0·5–2 ml., depending on the height of the ridges). Solutions are concentrated by standing the apparatus in front of a fan and dialysed by standing it in a beaker of dialysing solution; equilibrium is reached in 2–3 hr. without agitation. Solutions are expelled through the funnel by gently screwing the dialyser tubing at one of the ridges.

cedure was followed, olive oil alone being used. The animals were anaesthetized with ether 13–15 hr. after dosing, the vena cava was opened between the liver and the diaphragm, and the livers were perfused via the right ventricle with warm (40°) sodium chloride solution (0·15 M) for 20 min. or until no red colour remained. In spite of this a thin band due to red blood cells was always obtained in the tissue fractionations carried out immediately afterwards.

Tissue fractionation (see Hogeboom, Schneider & Striebich, 1952). The following operations were carried out below 5°. Perfused rat livers were washed in 0·25 M-sucrose solution, minced with scissors after removal of the larger blood vessels and homogenized one or two at a time for 90 sec. in a Potter–Elvehjem-type homogenizer in the presence of sucrose solution (30 ml. of 0·25 M). The homogenate was centrifuged for 8 min. at 800 *g* and the precipitate was resuspended in sucrose solution (30 ml. of 0·25 M), homogenized and centrifuged once more for 8 min. at 800 *g*. The two supernatant fractions were then combined and centrifuged for 4 hr. at 22 000 *g*. All products other than the final supernatant fraction were discarded.

Chromatography on ion-exchange celluloses. Diethylaminoethyl- (DEAE) and carboxymethyl- (CM) celluloses were prepared as described by Peterson & Sober (1956), highly purified cellulose fibres being used as the base.

The ion-exchange celluloses were thoroughly equilibrated against the starting buffer by suspending them in 0·1 M-buffer of the same pH as the starting buffer and then repeatedly filtering and resuspending them in the starting buffer. The final suspension was evacuated at a water pump to remove air bubbles, poured into the column and allowed to settle by gravity and filtration.

The final supernatant fraction prepared from two livers was dialysed against 2 l. of buffer solution (5 mM-phosphate, 5 mM-chloride, pH 7·9), concentrated to 20 ml. and dialysed once more against the same (used) buffer solution to reduce the volume to 20 ml. and bring it into equilibrium with 2 l. of buffer (5 mM-phosphate, 5 mM-chloride, pH 7·9). It was then chromatographed on DEAE cellulose as shown in Fig. 2 and the fractions shown in solid line were pooled, concentrated to 20 ml. and equilibrated by dialysis against about 200 ml. of 0·01 M-$H_2PO_4^-$, the volume of the latter being varied (depending on the volume of the protein solution before concentration) so as to give a final pH of 6·0. The product, which was yellow from treated rats and faint brown from controls, now contained a faint precipitate and was chromatographed on CM cellulose as shown in Figs. 3 and 4. The fractions shown in Figs. 3 and 4 were collected separately, concentrated to small volume (0·5–1·5 ml., depending on the size of the peak) and dialysed against the electrophoresis buffer (0·025 M-borate, 5 mM-chloride, pH 8·5). The DEAE first-peak proteins from one treated and one control liver were also concentrated to small volume and dialysed against the electrophoresis buffer.

Fractions C, D and E (Fig. 3) and c, d and e (Fig. 4) denatured rapidly and it was not possible to prepare concentrates of these without losing some material in this way.

Starch electrophoresis. The procedure has been fully described (Smithies, 1955). Gels were prepared from potato starch (12 g.; British Drug Houses Ltd.) that had been partially hydrolysed in the manner described by Smithies, and a buffer solution (100 ml. of 0·025 M-borate, 5 mM-chloride, pH 8·5) and were cast in Perspex trays with dimensions 23 cm. × 2 cm. × 0·6 cm. Slots 1–2 mm. wide were cut across the gels and filled with a supporting medium of dry starch grains previously floated in acetone to remove

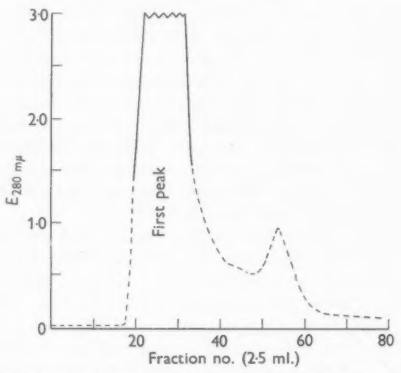

Fig. 2. Incomplete DEAE cellulose chromatogram of the supernatant fraction from two treated rat livers (20 ml. equilibrated against the starting buffer). Column, 20 cm. × 2·4 cm. (12 g.). Temperature, 2°. Flow rate, 30 ml./hr. Solvent: 5 mM-PO_4^{3-}, 5 mM-Cl^-, pH 7·9. Fractions under the solid line were combined to give the DEAE first peak. Untreated livers give the same graph.

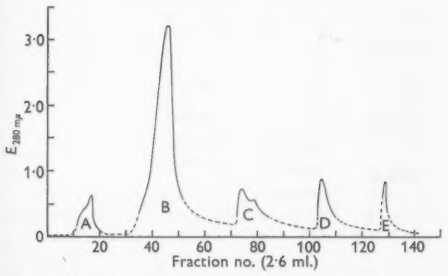

Fig. 3. CM cellulose chromatogram of the DEAE first-peak fraction (Fig. 2) from two untreated livers (15 ml. equilibrated against the starting buffer). Column, 13 cm. × 1·4 cm. (3 g.). Temperature, 2°. Flow rate, 30 ml./hr. Solvents: 0·01 M-PO_4^{3-}, 0·01 M-Cl^-, to fraction 23; 0·02 M-PO_4^{3-}, 0·02 M-Cl^-, to fraction 63; 0·03 M-PO_4^{3-}, 0·03 M-Cl^-, to fraction 95; 0·05 M-PO_4^{3-}, 0·05 M-Cl^-, to fraction 120; 0·1 M-PO_4^{3-}, 1·0 M-Cl^-, to fraction 143. All solvents were at pH 6·0. No gradients. Fractions under the solid lines were combined to give CM peaks A–E.

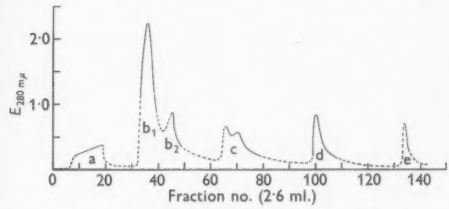

Fig. 4. CM cellulose chromatogram of the DEAE first-peak fraction (Fig. 2) from two treated livers (20 ml. equilibrated against the starting buffer). Column, 13 cm. × 1·4 cm. (3 g.). Temperature, 2°. Flow rate, 30 ml./hr. Solvents: 0·01 M-PO_4^{3-}, 0·01 M-Cl^-, to fraction 24; 0·02 M-PO_4^{3-}, 0·02 M-Cl^-, to fraction 56; 0·03 M-PO_4^{3-}, 0·03 M-Cl^-, to fraction 94; 0·05 M-PO_4^{3-}, 0·05 M-Cl^-, to fraction 127; 0·1 M-PO_4^{3-}, 1·0 M-Cl^-, to fraction 143. All solvents were at pH 6·0. No gradients. Fractions under the solid lines were combined to give CM peaks a–e.

the smaller particles. Concentrated protein solutions were then applied to the surface of the supporting medium by means of a capillary pipette and allowed to soak in until all the air had been displaced from the spaces between the grains. A potential of 15 v/cm., giving a current of 3–5 ma, was applied for 5 hr. during which time the whole system was kept at a temperature of 5°. As there was sometimes considerable smearing on the upper and lower surfaces of the gels, the latter were sliced in half horizontally before staining with Amido-Black 10 B, and, after excess of staining reagent had been washed out, the gels were soaked in water until they returned to their original size.

Protein bands containing azo dye were detected by soaking one strip in 15 % trichloroacetic acid solution for 1 hr. to bring out the pink colour of the dye, and afterwards comparing it with the other half of the gel which had been stained with Amido-Black 10 B.

Diagrams showing the patterns of bands obtained when various fractions were subjected to starch-gel electrophoresis are given in Fig. 5.

Estimation of azo dye bound to protein. Rapid qualitative estimations were made by heating 1 ml. of protein solution over a water bath until precipitation was complete. Trichloroacetic acid solution (1 ml. of 15 %, w/v) was then added and the precipitate developed a pink colour if dye was present.

For quantitative determinations, the dried protein sample (50–80 mg.) was extracted with ethanol (50 ml.) for 15 min. at 75°. After centrifuging and decantation, the precipitate was re-extracted twice with a boiling mixture of ethanol (30 ml.) and diethyl ether (10 ml.). The extracts were combined, concentrated to 5 ml., chilled, 5 ml. of chilled 7 N-hydrochloric acid solution was added and the extinction measured after 15 min. at 505 mμ in a 4 cm. cell. The blank correction was obtained after addition of 0·45 ml. of a saturated solution of stannous chloride in conc. hydrochloric acid to reduce the azo dye, and the difference between the two readings gives the amount of free dye present. The above method is that of J. Dijkstra & F. J. Joubert (in preparation).

The residue from the above-described extractions was dried under vacuum, a weighed amount was hydrolysed with potassium hydroxide solution and the hydrolysate was extracted with *n*-hexane to obtain non-polar bound dye and with diethyl ether to obtain polar bound dye. The procedure has been fully described by Miller & Miller (1947) and the only modification introduced was the addition of two drops of the stannous chloride solution at the end of each determination to get the blank correction. Results are given in Table 1.

DISCUSSION

Chromatography of soluble rat-liver proteins on DEAE cellulose (Fig. 2) gave a first-peak concentrate of neutral and basic proteins comprising about 30 % of the total soluble protein. This product was separated into five components by rechromatographing on CM cellulose (Fig. 3). A small amount

Table 1. *Azo-dye analysis of protein fractions from livers of rats treated with 4-dimethylamino-3′-methylazobenzene*

The DEAE first peak (see Fig. 2) contains all the supernatant liver proteins with which azo dye is associated as 'free' dye, 'polar bound' dye or 'non-polar bound' dye. Peaks a–e (see Fig. 4) are subfractions of this material.

Fraction		10^3 × concn. of dye (moles/100 mg. of protein)
DEAE first peak		20 (free dye)
		2 (non-polar bound dye)
		60 (polar bound dye)
Peak	a	+ (slight)
	b_1	−
	b_2	−
	c	−
	d	+
	e	+

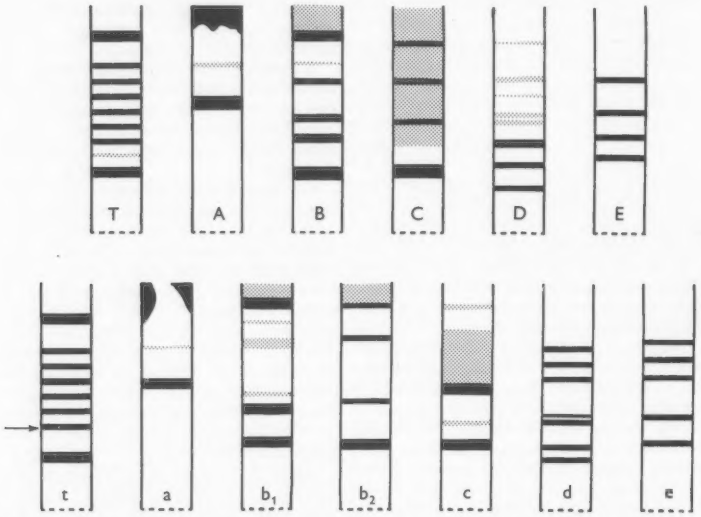

Fig. 5. Diagram showing the patterns produced by starch-gel electrophoresis of protein fractions from treated and untreated rat livers after staining the gels with Amido-Black 10B. Only that section of the gel between the sample slot (top) and the cathode (broken line) is shown, although components which moved slowly in the direction of the anode were also present in some cases. Stippling denotes fainter bands and areas of stippling indicate background absorption strong enough to obliterate such bands. Gels t and T were obtained from the DEAE first peaks of treated and untreated livers respectively (see Fig. 2); the band bearing the azo dye is marked with an arrow. Gels A–E were obtained from the corresponding untreated CM peaks A–E (Fig. 3) and gels a–e from the corresponding treated CM peaks a–e (Fig. 4).

of material was always irreversibly adsorbed on to CM cellulose columns and peaks showed a tendency to trail, with the result that some of the later peaks contain material also present in earlier ones. Use of a cation-exchange resin having groups less acidic than carboxyl may enable these difficulties to be overcome.

The preparative procedures described above gave little indication of the degree of separation actually achieved, and a sensitive method was required for the qualitative analysis of complex mixtures of proteins. Starch-gel electrophoresis was found to be very satisfactory for this purpose and gave reproducible results on small amounts of material. All the starch-gel bands discussed in this paper migrate in the direction of the cathode.

Starch-gel electrophoresis of the DEAE first peak (Fig. 2) gave a pattern of eight distinct bands (Fig. 5), the slowest of which is probably an artifact produced by fractionation on DEAE cellulose, since it does not appear in starch-gel patterns of the total soluble liver proteins. When the DEAE first-peak proteins were rechromatographed on CM cellulose, starch-gel electrophoresis of the resulting peaks showed that the material has been separated into several different groups of proteins. However, the bands given by peaks A–E cannot all be correlated with the bands given by the DEAE first peak from which they were obtained.

The authors consider that use of the separative procedures in sequence gives a greater degree of resolution than any previously obtained. It is necessary to emphasize that the DEAE first-peak proteins to which this investigation was confined are only a small group of the total liver proteins.

Soluble liver proteins from rats which had been fed with the carcinogenic azo dye 4-dimethylamino-3′-methylazobenzene a few hours previously were fractionated by the procedures discussed above. Chromatography on DEAE cellulose gave the same separation as with the untreated liver proteins; protein-bound dye was found only in the first peak. A complete DEAE chromatogram of the soluble liver proteins from a treated liver is shown in Fig. 6. In Fig. 6 the two peaks of Fig. 2 were eluted as a single first peak, and this was the only peak to contain protein-bound dye.

Starch-gel electrophoresis of the DEAE first-peak proteins from treated livers gave a pattern in no way significantly different from that given by

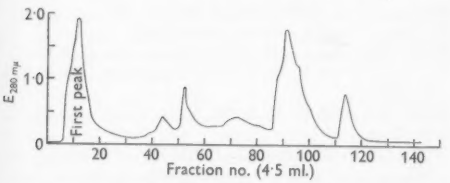

Fig. 6. Complete DEAE cellulose chromatogram of the supernatant fraction from one treated rat liver (20 ml. equilibrated against the starting buffer by dialysis). Column, 13 cm. × 1·4 cm. (3 g.). Temperature, 2°. Flow rate, 54 ml./hr. Solvents: 5 mM-PO_4^{3-}, 5 mM-Cl^-, pH 7·9, to fraction 30; 0·05 M-PO_4^{3-}, 0·05 M-Cl^-, pH 5·9, to fraction 80; 0·1 M-PO_4^{3-}, 1·0 M-Cl^-, pH 4·5, to fraction 145. Solvents were run through a mixing chamber (hold-up volume 150 ml.) to provide a gradient, so the final concentrations were never reached.

untreated livers (see Fig. 5). The band marked with the arrow was the only band in which azo dye could be detected.

When the DEAE first-peak proteins from a treated liver were rechromatographed on CM cellulose, a separation was obtained identical with that obtained with the corresponding untreated material, except that the single peak B was now replaced by the two peaks b_1 and b_2 (compare Figs. 3 and 4). Neither b_1 nor b_2 contained any bound azo dye, nearly all of which was found in peaks d and e.

The patterns obtained after starch-gel electrophoresis of the various CM peaks are shown in Fig. 5. Peaks b_1 and b_2 appear to contain the same components present in the corresponding peak B from the untreated liver, but one of these is present only in b_1 and others are largely concentrated in b_2. Peaks d and e from the treated livers were extremely unstable and attempts to develop azo-dye bands after starch-gel electrophoresis were unsuccessful; the only colour obtained was in the vicinity of the slot, indicating that the dye protein had denatured in the early stages of electrophoresis.

Since in most other respects the DEAE first-peak proteins from treated and untreated livers had almost identical behaviour, it seems that the majority of these proteins were not affected by administration of the carcinogen. It is significant that, in spite of the extensive fractionation carried out, there was no evidence at any stage to show that the dye was bound to more than one component, and this supports the contention that binding of azo dye to liver protein is highly specific.

Sorof *et al.* (1958), who carried out a very efficient fractionation of the so-called 'h' proteins from treated rat livers and obtained six sub-components, did in fact find that two of these contained bound azo dye. We hesitate to draw any comparisons between our results and those of Sorof *et al.* because we do not know how far our DEAE peak corresponds to their 'h' proteins. Furthermore, we have confined ourselves to the effects of a single 10 mg. dose of carcinogen over a few hours, whereas Sorof *et al.* used a continuous dose (about 5 mg./day) over a period of $2\frac{1}{2}$ weeks, and it is possible that other changes occur in the protein composition of the cell during this time. As the half-life of the protein-bound dye is only 4 days (Gelboin *et al.* 1958) a considerable amount of dye already bound to protein must have been metabolized during this period.

Our results show that in addition to dye-binding there is an immediate qualitative change in a group of soluble liver proteins that do not bind the dye. This has not been shown before.

SUMMARY

1. Soluble rat-liver proteins were fractionated in sequence by anion- and cation-exchange chromatography and by starch-gel electrophoresis.

2. A fraction corresponding to the so-called 'slow-moving proteins' was resolved into eight components.

3. Only one of these components contained covalently bound azo dye when the carcinogen 4-dimethylamino-3′-methylazobenzene was fed to the animals shortly before they were killed.

4. The properties of another component were found to have changed as a result of feeding with the dye.

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Carbon Dioxide Fixation in *Trichomonas vaginalis*

BY R. WELLERSON, G. E. DOSCHER AND A. B. KUPFERBERG
Ortho Research Foundation, Raritan, New Jersey, U.S.A.

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Reports of carbon dioxide fixation by non-chlorophyll-containing protozoa are rarely encountered in the literature. With the exception of a report by Kupferberg, Singher, Lampson, Levy & Romano (1953) on *Trichomonas vaginalis*, all other studies have been confined to *Tetrahymena* (van Niel, Thomas, Ruben & Kamen, 1942; Ryley, 1952; Lynch & Calvin, 1952) and *Trypanosoma* (Searle & Reiner, 1940; Moulder, 1948).

Kupferberg *et al.* (1953) demonstrated that *T. vaginalis* fixed carbon dioxide during growth and that the product was almost entirely outside the cell. This material isolated from the culture medium served as the starting material for this study. The ^{14}C-labelled product has been isolated and identified and the site of the radioactive carbon determined.

METHODS

Fixation material. The entire investigation was conducted with the $^{14}CO_2$-fixation product isolated by Kupferberg *et al.* (1953). This material had been freeze-dried and stored in a closed container at 5°.

Chromatographic procedures. The freeze-dried material was reconstituted with water, passed through an Amberlite IR-120 cation-exchange column (1·1 cm. × 16 cm.) in the hydrogen form, and washed from the column with water at the rate of 14–16 drops/min. The material was prepared for chromatography by concentration of the eluate *in vacuo*. The dried material was resuspended in water (0·5–1·0 ml.) and was spotted or streaked onto Whatman no. 1 filter paper. The chromatograms were run ascending at 26°, either the ethyl ether–acetic acid–water (13:3:1, by vol.) system of Denison & Phares (1952) or a butanol–88% formic acid (95:5, v/v) system saturated with water, being used. The former system was used initially for a rapid differentiation of organic acids whereas the latter was employed when better definition of lactic acid and malic acid was required. The chromatograms were air-dried and then steamed gently while being exposed to the heat of an infra-red lamp, as described by Denison & Phares. The acids were identified on the paper chromatogram as yellow spots on a purple background after spraying with bromophenol blue (0·045 g./100 ml. of ethanol, pH 7·0). The location of the radioactivity on the paper chromatograms was determined either by placing the chromatogram in direct contact with X-ray film (Kodak Ltd. No-Screen) for 31 days, or by dividing it into small segments (5 mm. × 25 mm.) and making direct counts on each section with a windowless Geiger–Müller counter.

Preparation for infrared spectrophotometry. To accumulate sufficient material for the infrared studies, large sheets (50 cm. × 50 cm.) of Whatman no. 1 filter paper were streaked with the concentrated material along the length of the origin. The chromatograms were run ascending at 26° with the butanol–formic acid system. The location of the acids was established by cutting a thin vertical strip from each edge and a third from the centre and spraying them with the indicator. The acids were eluted from the unsprayed chromatogram with water, each zone being eluted several times with a total volume of 100 ml. Each eluate was filtered through Whatman no. 2 filter paper, concentrated *in vacuo* and the residue treated several times with 95% ethanol. The ethanol solutions were pooled and concentrated and the insoluble residue was discarded. The concentrated material was placed between KCl crystals and the infrared spectrum was determined in a Baird infrared spectrophotometer.

Degradations studies. Material to be degraded was obtained in the same manner as that for the infrared determinations. Lactic acid, with and without carrier, was degraded according to the procedure of Elsden & Gibson (1954), and the aeration technique was used. The aldehyde was trapped as the bisulphite complex and the carbon dioxide was trapped in NaOH. The carbonate was precipitated as $BaCO_3$ by the addition of a saturated solution of $BaCl_2$. Self-absorption corrections were made as described by Schweitzer & Stein (1950).

Enzymic studies. Cell-free extracts of *T. vaginalis* were prepared as described by Wellerson, Doscher & Kupferberg (1959), except that the one used for the carboxylase determination was neither washed nor frozen.

Carboxylase activity was tested for manometrically according to the procedure of Singer (1955). Glucose 6-phosphate-dehydrogenase activity was measured by following the reduction of the coenzyme at 340 mμ in a Beckman DU spectrophotometer. Phosphoriboisomerase activity was determined by the Dische & Borenfreund (1951) cysteine–carbazole test for ketopentose.

EXPERIMENTAL

Identification of radioactive product. The radioactive material was not effectively adsorbed by the cation-exchange column since 96–100 % recovery was obtained when the column was washed with a small volume of water. Three acids were detected on paper chromatograms of the concentrated aqueous eluate. Lactic acid and malic acid were identified by comparing their R_F values with those of known standards with several solvent systems. The third acid had R_F 0·1–0·25 when run for 16 hr. in butanol–formic acid–water. The radioactivity was located entirely in the area occupied by the lactic acid. Final confirmation of the identity of the $^{14}CO_2$-fixation product was obtained by comparison of the infrared spectrum of the radioactive material eluted from the chromatogram with that of lactic acid (Fig. 1).

The eluate of the radioactive area of a paper chromatogram was degraded to establish the location of the ^{14}C in the lactic acid molecule. In the procedure used (Elsden & Gibson, 1954) the carbon dioxide produced comes entirely from the carboxyl group. Table 1 shows that all of the tracer was located in the carboxyl carbon of the lactic acid.

Enzymic experiments. The absence of any label in the malic acid isolated eliminates the carboxylation of pyruvate as the primary mechanism of fixation. A preliminary study of the role of carboxylase or ribulose diphosphate in the fixation of carbon dioxide by this protozoan was made by examining certain essential enzymic reactions. Carboxylase, an enzyme common to bacteria, fungi and plants, catalyses the following reaction:

$$\text{pyruvate} \xrightarrow[Mg^{2+}\text{ ion}]{\text{cocarboxylase}} \text{acetaldehyde} + CO_2.$$

By the manometric procedure of Singer (1955) the carboxylase activity of a freshly prepared cell-free extract was examined. There was no carbon dioxide liberated during the course of 120 min., in spite of the addition of both cocarboxylase and Mg^{2+} ions to the reaction mixture. A number of extracts of *T. vaginalis* were examined; all were negative for carboxylase.

Since ribose metabolism is associated with the fixation of carbon dioxide in certain bacteria and plants, certain of the steps involved in the synthesis of the key compound, ribulose diphosphate, were studied: glucose 6-phosphate dehydrogenase, because it catalyses the initial step in the conversion

Table 1. *Degradation of lactic acid*

Reaction flask contained 1 ml. of zinc lactate (55 μmoles) in N-H_2SO_4, 3 ml. of [^{14}C]lactic acid eluted from the chromatogram (6167 counts/min./ml.), 5 ml. of $Ce(SO_4)_2$ reagent. Bisulphite trap contained 5 ml. of $NaHSO_3$ and the alkali traps 25 ml. and 5 ml. of 0·1 M-NaOH.

Vessel	Total radioactivity* (counts/min.)
Reaction flask (start)	18 500
Reaction flask (finish)	0
Bisulphite trap	0
Alkali traps	16 700

* Corrected for self-absorption.

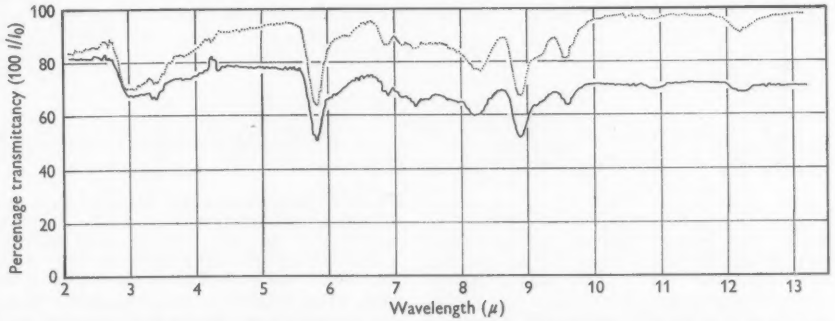

Fig. 1. A comparison of the infrared spectra of the fixation material and authentic lactic acid. Broken line, known lactic acid; continuous line, eluate from chromatogram (R_F 0·6).

of hexose into pentose; phosphoriboisomerase, for the isomerization of ribose 5-phosphate.

Glucose 6-phosphate dehydrogenase catalyses the reaction:

$$\text{glucose 6-phosphate} + \text{TPN}^+ \rightleftharpoons \text{6-phosphogluconate} + \text{TPNH} + \text{H}^+.$$

The reduction of the triphosphopyridine nucleotide (TPN) produces an absorption maximum at 340 mμ which allows the above reaction to be followed spectrophotometrically. The addition of 10 μmoles of glucose 6-phosphate to the cell-free extract produced a rapid reduction of TPN but did not reduce diphosphopyridine nucleotide (DPN) (Fig. 2). Inorganic phosphate, a known inhibitor of this enzyme, when present at a concentration of 0·06 M inhibited the reduction of TPN.

The extract was tested for phosphoriboisomerase activity with both ribose and ribose 5-phosphate as substrates. Portions were removed periodically and examined for the presence of ketopentose by the cysteine–carbazole reaction (Fig. 3). Only when ribose 5-phosphate was present was ketopentose produced. Both the ribose system and the endogenous control produced a slight decrease in E at 540 mμ. Because this particular extract did not possess an active phosphohexokinase, the decrease in E noted in the ribose 5-phosphate system after 60 min. indicates that the cysteine–carbazole colour resulted from ketopentose and not ketohexose.

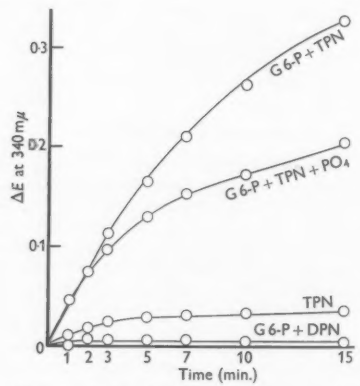

Fig. 2. Glucose 6-phosphate-dehydrogenase activity of *T. vaginalis*. Each cuvette contained: cell-free extract (equivalent of $5{\cdot}2 \times 10^7$ cells); 2-amino-2-hydroxymethyl-propane-1:3-diol buffer, pH 7·7, 600 μmoles; $MgSO_4$, 1 μmole. Glucose 6-phosphate (G 6-P; 10 μmoles), TPN or DPN (0·77 μmole) and Na_2HPO_4 (200 μmoles) were added as shown. The total volume was 3 ml. and incubation was at 26°.

DISCUSSION

Succinic acid has been reported as playing a major role in the fixation of carbon dioxide by both *Tetrahymena* (Lynch & Calvin, 1952) and *Trypanosoma* (Searle & Reiner, 1940). Sixty-three per cent of the labelled carbon dioxide was fixed in the form of succinate by *Tetrahymena*. The fact that no labelled succinate was isolated from *Trichomonas vaginalis* and that lactic acid accounts for nearly all the carbon dioxide fixed suggests that the mechanisms involved are different. The identification of a carboxyl-labelled lactic acid as the end product of carbon dioxide fixation eliminates the malic acid dehydrogenase or 'malic' enzyme system as possible routes of fixation. Failure to

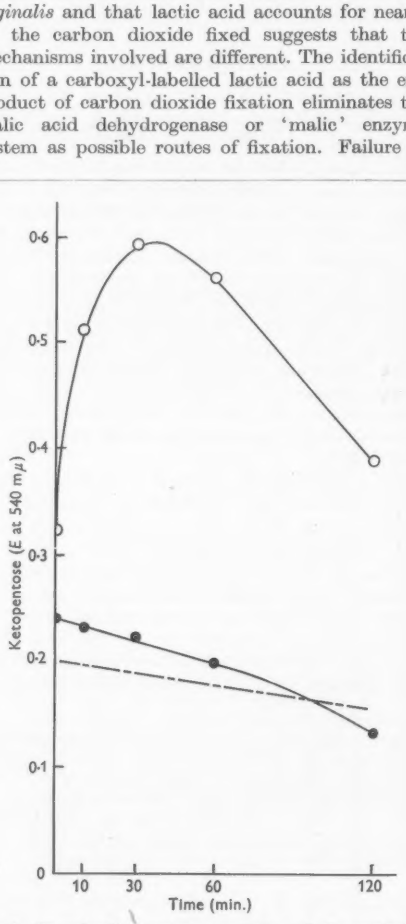

Fig. 3. Phosphoriboisomerase activity of *T. vaginalis*. The reaction mixtures contained: cell-free extract (equivalent of $1{\cdot}25 \times 10^8$ cells), M/15 phosphate buffer, pH 7·0, and ribose 5-phosphate (7·2 μmoles) or ribose (6·6 μmoles). The control was without substrate. Volume of the reactants, 15 ml. Incubation temperature, 37°. Ketopentose was determined by the cysteine–carbazole method of Dische & Borenfreund (1951). ○, Ribose 5-phosphate; ●, ribose; — — —, control.

demonstrate carboxylase activity also excludes the carboxylation of acetaldehyde as a means of fixation. One route to which the data do seem to apply is that of a pentose diphosphate carboxylation. This mechanism has generally been associated only with photosynthetic cells. Santer & Vishniac (1955). Trudinger (1956), Aubert, Milhaud & Millet (1956) and Suzuki & Werkman (1958) described this type of fixation as occurring in chemoautotrophic bacteria of the genus *Thiobacillus* and found that both whole cells and cell extracts formed phosphoglyceric acid when supplied with carbon dioxide and ribulose diphosphate. According to the enediol mechanism suggested by Calvin (1954) 3-phosphoglyceric acid resulting from a carboxylation with $^{14}CO_2$ would contain ^{14}C only in the carboxyl carbon atom. A carboxyl-labelled 3-phosphoglyceric acid therefore, if metabolized via the Embden–Meyerhof route, should produce carboxyl-labelled lactic acid.

The presence of glucose 6-phosphate dehydrogenase and phosphoriboisomerase activity as well as the evidence for an Embden–Meyerhof system in this organism (Wirtschafter & Jahn, 1956) lend support to the suggestion of the existence of such a system.

SUMMARY

1. The end product of the fixation of $^{14}CO_2$ by *Trichomonas vaginalis* was characterized by chromatography and by its infrared spectrum as lactic acid.

2. The ^{14}C occurred only in the carboxyl group of lactic acid.

3. Extracts of the organism contained glucose 6-phosphate dehydrogenase and phosphoriboisomerase, but no carboxylase.

The authors wish to acknowledge the able technical assistance rendered by Miss Judith Kahn and Miss Dorothea Polhemus.

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Phospholipid Metabolism in Nervous Tissue

2. METABOLIC STABILITY*

By A. N. DAVISON and J. DOBBING
Department of Pathology, Guy's Hospital Medical School, London, S.E. 1

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Davison & Dobbing (1959) put forward a provisional hypothesis that the brain constituents, in common with the constituents of other tissues, may be divided into at least two main groups according to their biological half-lives. Evidence was presented for the possible existence of a phospholipid compartment in the brain whose metabolic stability was much greater than could be accounted for by the known turnover rates of even its most slowly metabolized components. A possible explanation was that phospholipids of certain anatomical structures, for example the myelin sheaths, were metabolically inert, whereas in other parts of the brain these same phospholipids were metabolic-

* Part 1: Davison & Dobbing (1959).

ally active. This seemed to reconcile a current view, that brain phospholipids were rapidly metabolized at a rate comparable with that in other tissues, with our own findings with [3-^{14}C]serine and ^{32}P that labelled phospholipid molecules were still to be found in substantial quantity long after such activity had disappeared from other organs (Davison, Morgan, Wajda & Wright, 1959; Davison & Dobbing, 1959).

Much of the central nervous system, including the myelin sheath, undergoes no morphologically detectable change once maturity is reached, and there is virtually no possibility of new formation of neurons or myelin sheaths during adult life even when these structures are damaged by disease. Thus it is predictable on the basis of our hypothesis that it should be possible to demonstrate a non-structural, metabolically labile group of brain phospholipids in both young and adult animals; but that the metabolic inertness already shown when [3-^{14}C]serine is given to developing animals should not be demonstrable by the injection of isotopes into adults. Such a group of structural, stable constituents could, moreover, only be distinguished in long-term *in vivo* experiments in which they had been labelled during the course of myelinogenesis (Davison, Dobbing, Morgan & Wright, 1958, 1959; Davison, Morgan, Wajda & Wright, 1959).

The experiments presented here were designed to investigate this aspect of the problem and to determine to what extent metabolic inertness could be demonstrated in brain phospholipids by the use of ^{32}P.

METHODS

Two groups of white rats of different ages received an intraperitoneal injection of ^{32}P as phosphate in 0·9% sodium chloride soln. and were eventually killed by chloroform. In the first group, 19 rats (two litters) each received 25 μc of ^{32}P in 0·25 ml. of 0·9% sodium chloride soln. on the sixteenth postnatal day, when they weighed 30–35 g. They were reared in identical fashion and killed in pairs at intervals up to 116 days, and thereafter singly up to 189 days after injection. In the second group, seven adult rats of the same strain weighing 300–400 g. each received 1 μc of ^{32}P/g. body wt. as a solution containing 1 μc/ml. of 0·9% sodium chloride. The second group were killed singly at intervals up to 179 days after injection. All animals in both groups survived the rather high initial injection without any apparent ill effects. The developing series gained weight at a normal rate.

A third group of seven rats of the same litter each received 2 μc of [1-^{14}C]glycerol intraperitoneally on the thirteenth post-natal day, and a fourth group of adult rats of the same strain were each injected with 10 μc of [1-^{14}C]-glycerol by the same route. These two groups were killed at intervals up to 110 days after injection.

Extraction procedures. Extraction of total lipid from brain, spinal cord and sciatic nerve by 20 times their volume of chloroform–methanol (2:1, v/v), together with the trichloroacetic acid extraction of acid-soluble phosphorus from the blood, were by the conventional methods previously described (Davison & Dobbing, 1959).

Determination of ^{32}P radioactivity. Radioactivity of suitably diluted lipid extracts from the first two groups of animals was determined in a G.M. β-counter for liquids until 96 days after the injection of ^{32}P. After this time counting was continued in an Ekco type N612 liquid scintillation counter operated at room temperature, with an a.c. supply stabilized to $\pm$0·25% by a Servomex a.c. stabilizer. A Dynatron N101 single-channel discriminator was employed which reduced the background of the counter to 14·3–18·2 counts/min. (see Table 1). The amplifier was used at a low-gain setting of $\times$50.

The solvent used for counting was xylene (reagent grade, May and Baker Ltd.) with 2:5-diphenyloxazole as scintillator solute. Suitable portions of lipid extracts (up to about 30 ml. of brain extract) were evaporated to dryness and the residue was dissolved in xylene plus scintillator. Background counts were first determined on 20 ml. of xylene plus scintillator in a sample container, by repeated 5 min. counts, up to 15 times. The dried lipid extract was then dissolved in the same xylene plus scintillator and the radioactivity redetermined in the same sample container for an identical time. This procedure was necessitated by the finding that different sample containers gave different background counts (13·5–20·6 counts/min.; see Table 1). In addition, it was shown that the performance of the scintillation-counting equipment was stable throughout the lengthy period of the experiment, a background count being made on each occasion, with the same preparation of solvent and scintillator in a standard container (see 'Standard background', Table 1).

To correlate radioactivity determined in the liquid β-counter with that in the scintillation counter, several high-activity samples were counted by both methods. It was found that the scintillation technique gave a count which was constantly six times as high, and the results determined by this technique were therefore divided by six. The background counts for both methods were of a similar order.

Determination of ^{14}C radioactivity. Plasma was treated with 15 times its volume of acetone–ethanol (1:1, v/v). After being filtered, the extract was dried and the residue suspended in xylene and phosphor, and the radioactivity determined in the scintillation counter. The ^{14}C activities of whole-brain lipids and protein-free lipids were also determined by scintillation counting as previously described (Davison, Morgan, Wajda & Wright, 1959).

RESULTS

The whole-brain counts of phospholipid ^{32}P for 189 days after injection of inorganic ^{32}P into neonatal animals are shown in Fig. 1, together with the blood levels of trichloroacetic acid-soluble ^{32}P for the first 50 days of this period. No counts were made until the eleventh day after injection because events in the early stages had already been investigated (Davison & Dobbing, 1959). From 11 days onwards the pattern of the decline in brain-phospholipid ^{32}P has two main components: a steady rate of loss until about 100 days after in-

jection, after which time there is no demonstrable decline. The part of the curve drawn to represent lipid levels of radioactivity during the second half of the experiment is the calculated best straight line through the relevant points (96–189 days). The slope of the regression line (16 ± 12 counts/min./day) is consistent with the points falling on a horizontal line. Acid-soluble phosphorus had disappeared from the blood long before this second, stable component appeared in the results. A similar pattern is discernible in Fig. 2, which shows the persistence of phospholipid ^{32}P in the spinal cords and sciatic nerves of the same series of young animals. The small size of the sciatic-nerve sample gave rise to a greater degree of experimental error in the later samples of low activity, and for this reason it was found impracticable to investigate the radioactivity of peripheral nerve after 150 days.

The demonstration of the complete stability of a

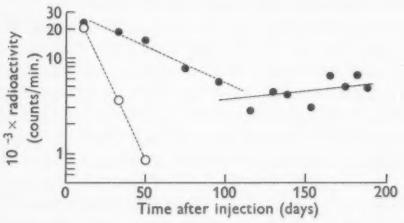

Fig. 1. Radioactivity in phospholipids of whole brain (●) and acid-soluble phosphorus of blood (○). Rats were injected with ^{32}P when 16 days old. Radioactivity in blood is expressed as $10^{-3} \times$ counts/min./10 ml. of trichloroacetic acid extract. The continuous line is the best straight line calculated by the method of least squares for the points 96–189 days. See Table 1 for standard errors.

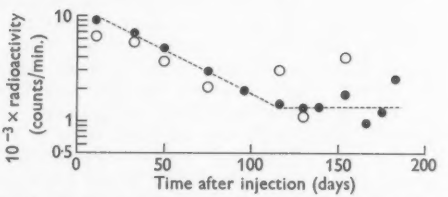

Fig. 2. Radioactivity in phospholipids of rat spinal cord (●) and sciatic nerve (○). Rats were injected with ^{32}P when 16 days old. Radioactivity is expressed as $10^{-3} \times$ counts/min./mg. of phosphorus. The relative standard errors increased from 1 to 28% for the spinal cord and from 3 to 50% for the sciatic nerve during the course of the experiment.

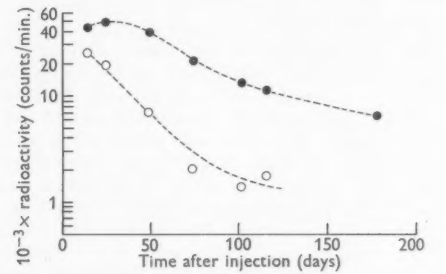

Fig. 3. Radioactivity in phospholipids of whole brain (●) and acid-soluble phosphorus of blood (○) of adult rats previously injected with ^{32}P. Radioactivity is expressed as $10^{-3} \times$ counts/min./whole brain. Radioactivity in blood is expressed as $10^{-3} \times$ counts/min./10 ml. of trichloroacetic acid extract. The relative standard errors increased from 1 to 18% during the course of the experiment.

Table 1. *Radioactivity of brain-lipid extracts*

A liquid β-counter was used up to 96 days after injection; thereafter radioactivity was determined by scintillation counting as described in the text.

Time after injection (days)	Time each sample counted (min.)	Counts/min.				Total corrected brain-lipid radioactivity (counts/min./whole brain)	Standard error (counts/min./whole brain)
		Standard background	Background for sample container	Sample count	Standard error		
96	40	—	13·8*	13·2	1·01	5390	412
96	40	—	11·8*	13·9	0·97	4780	334
116	20	14·3	15·6	30·4	1·75	2665	153
116	20	15·0	13·5	27·2	1·65	2840	172
130	60	18·0	15·5	24·7	0·97	4330	170
139	40	14·6	14·7	14·5	1·05	4070	294
154	40	15·4	15·4	9·8	1·01	2990	308
166	50	18·2	20·6	7·04	0·96	6450	882
175	50	15·3	19·5	4·18	0·93	4920	1100
183	50	14·8	17·7	4·04	0·89	6470	1430
189	75	18·1	20·2	1·69	0·75	4880	2160

* Background count in the G.M. β-counter tube.

proportion of the brain phospholipids depends greatly on the validity of the counting procedure during the last 80 days of the experiment. Table 1 expresses these particular results in more detail. The actual counts obtained on the brain-lipid extracts and their associated backgrounds are shown, together with the standard error of the samples (S.E. $\sqrt{[2B+S]/t}$, where B is total background count, S is total sample count, t is time; background and sample being counted for equal periods).

The changing levels of whole-brain-phospholipid ^{32}P in the adult animals are shown in Fig. 3, together with the values for trichloroacetic acid-soluble ^{32}P in whole blood. Unlike the results with the young animals, the adult-brain phospholipid has not achieved its maximum radioactivity 15 days after injection, but when it does so it subsequently declines at a rate very similar to that of the more labile phospholipids of the immature brain. The pattern of blood radioactivity in the older group, however, contrasts very markedly with that in the young. The level in the adult blood is at all times much higher. Fifty days after injection it is nearly eight times as high, owing to the more rapidly declining radioactivity in the young, and a quite unexpected persistence in the adult blood is demonstrable up to at least 116 days after injection. There is no evidence from these results of any complete metabolic inertness in the brain phospholipids of this series.

After injection of [^{14}C]glycerol into young and old rats, the blood radioactivity fell within a week to negligible values in both cases. When expressed in terms of whole-brain activity, comparable and maximal amounts of ^{14}C were incorporated into brain lipids of the two groups on the first day (Fig. 4). The rate of decline of activity in the old rats was, from the beginning of the experiment, however, consistently much greater than that in the young.

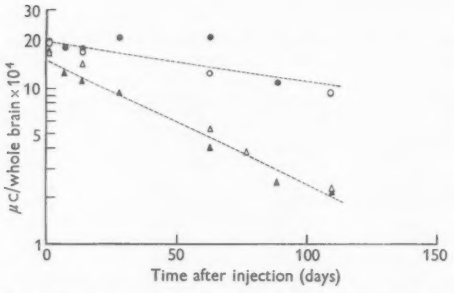

Fig. 4. Radioactivity in whole-brain lipids of young and adult rats injected with [1-^{14}C]glycerol. Rats were injected with [1-^{14}C]glycerol when 13 days old. Radioactivity of whole-brain-lipid extract (●) and protein-free lipid (○) are recorded. Results for adult rats are whole-brain-lipid extract (▲) and protein-free lipid (△) multiplied by two.

DISCUSSION

The most important conclusion to be drawn from these results is that a measurable proportion of the brain-phospholipid phosphorus, as is shown in Fig. 1, possesses a high degree of metabolic stability which may indeed be virtually complete. This apparently stable compartment could not have been revealed in an experiment lasting much less than 6 months: it did not even appear in our earlier, 70-day, experiment (Davison & Dobbing, 1959) because it is obscured for about 100 days under these conditions by the incorporation of ^{32}P into other, more labile, phospholipids. The extent to which this finding supports a hypothesis of metabolic inertness of the chemical constituents of the myelin sheath must now be discussed.

Complete proof for the hypothesis demands the demonstration of stability in all the differing constituent molecules that comprise the anatomical structure. So far this has been accomplished in similar experiments for the cholesterol molecule by the use of [4-^{14}C]cholesterol (Davison *et al.* 1958; Davison, Dobbing, Morgan & Wright, 1959), and for those parts of certain phospholipids as well as cerebrosides and cholesterol into which the ^{14}C of [3-^{14}C]serine was incorporated in developing animals. The present results suggest the same principle for phospholipid ^{32}P. The possibility must of course, be considered that a dynamic state, exists internally within the brain, or within the myelin sheath, in such a way as not to be revealed by this type of experiment. If this were so, however, the metabolic processes involved would have to take place by means of intermediates which did not themselves leak into the circulation, and the efficiency of re-incorporation into phospholipid would have to be complete. 'Internal turnover' in this sense remains a possibility, but seems most unlikely. ^{14}C and ^{32}P can both be incorporated in permanent fashion into brain phospholipid, although strictly, of course, this remains to be demonstrated simultaneously in the same molecules. Further support for the hypothesis may be derived from the evidence that the persisting [4-^{14}C]-cholesterol is mainly located in the white matter (Davison, Dobbing, Morgan & Wright, 1959) and that the same is true of the persisting phospholipid ^{32}P (Davison & Dobbing, 1960).

Our findings must also be discussed in the light of those of Thompson & Ballou (1954, 1956). These authors studied the biological half-lives of the components of a large number of tissues by means of long-term experiments with tritium as an indi-

cator, and, in their earlier paper, first demonstrated a general stability of brain phospholipids. Later they were able to show that biological half-lives, although presenting a wide range of values, may be divided grossly into two main groups. Thus more than half of the total brain constituents of rats exposed to tritium during their development have an average half-life as long as 150 days; and of the phospholipids in the entire body, one-third have an average half-life of about 220 days. Of the tissues used collagen was the only one that appeared to be virtually completely inert. Our approach differs essentially from theirs in being a more specific study of brain phospholipids. Presumably the main reason for the non-appearance in their figures of such stability of brain phospholipids as we have demonstrated is that they have inevitably included all the brain constituents labelled with tritium, many of which have very great metabolic activity, and by taking a single composite figure the stability of some of them has been obscured. Both their 'long-lived' tissue component and their 'shorter-lived' components would be included in our 'labile' group. The metabolically inert group of brain lipids is, in these terms, a third, 'permanent' component and is to be compared with collagen and possibly actomyosin whose inertness appeared likely to be 'not an intrinsic property of the molecules themselves, but an expression of the morphological stability of the fibrous structures of which these proteins form a part' (Henriques, Henriques & Neuberger, 1955).

A necessary corollary of the inertness of these anatomical structures within the brain is that it should be possible to incorporate injected material into them only during the course of their development, and not into the adult structure. Fig. 3 shows the results of an attempt to investigate the long-term fate of phospholipid ^{32}P after the injection of ^{32}P into adults. At first sight it has not been convincingly shown that there is no ultimate plateau in the curve, but several of its features merit further discussion. Prominent amongst these is the very much greater persistence, both in quantity and duration, of the acid-soluble ^{32}P in the blood of the adults compared with the young. Measurable and significant quantities of precursor ^{32}P in the blood are being presented for synthesis into brain phospholipids as long as 116 days after injection, long after the acid-soluble ^{32}P in the blood of the immature series of animals has declined to negligible values. The old and the young can be more usefully compared for these purposes by taking these differing blood levels into account, as can be seen from Table 2 in which the results are expressed as the ratios of whole-brain phospholipid to blood concentration of acid-soluble ^{32}P in each series. In the young the ratio rapidly approaches infinity as the blood levels approach zero, whereas in the adults the values for the ratio rise more slowly and then begin to decline. This subsequent decline would be unlikely to forshadow an impending plateau in brain concentrations. The apparent similarity in the rate of decline of adult and young brain-phospholipid ^{32}P is therefore fortuitous and may be considered to be attributable to the greater persistence of precursor in the blood of the former. The same factor may equally well account for the delay in achieving maximum labelling of brain phospholipids in the adult series compared with the young. These results illustrate the importance of estimating blood levels of precursor in all experiments of this kind. With regard to ^{32}P, the differences of persistence of this in blood are probably largely due to the influence of the skeleton, which in both series takes up a high proportion of the injected dose. In the developing animals most of the phosphorus incorporated into the bones is retained, whereas in the adults a similar amount is slowly liberated into the circulation during the ensuing months after injection, maintaining a significant level of availability of precursor phosphorus for phospholipid metabolism (unpublished observations).

Table 2. *Whole-brain phospholipid ^{32}P expressed in terms of blood acid-soluble ^{32}P concentration, after injection of ^{32}P into neonatal and adult rats*

Details of methods used are given in the text.

Neonatal rats		Adult rats	
Time after injection (days)	Brain-phospholipid ^{32}P / Blood-acid-soluble ^{32}P	Time after injection (days)	Brain-phospholipid ^{32}P / Blood-acid-soluble ^{32}P
11	11·5	15	17·5
		25	25·5
33	52	—	—
50	175	49	55
75	∞	74	100
		102	97
		116	63·5

The experiments with [1-^{14}C]glycerol injected into both neonatal and adult rats were undertaken to test the influence of surviving blood levels of isotope on the labelling of brain lipids. Glycerol is predominantly incorporated by guinea-pig cortex *in vitro* into phosphoglycerides (Hokin & Hokin, 1958), and only to a small extent into non-phosphorus-containing lipids. In the present experiments the blood level of ^{14}C 1 week after injection was negligible in both series, and may therefore be presumed to have had a similar influence on the degree of incorporation into brain lipids in each group. Fig. 4 shows that, from the outset, the rate of decline of activity in the brains of the younger series is much slower than that in the old, and presents a picture more in keeping with the findings of Thompson & Ballou (1956). The experiment did not continue long enough, however, to demonstrate the presence or otherwise of stable components.

As can be seen from Fig. 2, the spinal cord and peripheral nerve show the same extended persistence of phospholipid ^{32}P when the isotope is administered to neonatal animals as has been shown in brain.

In conclusion, the suggestion that there exists in nervous tissue some anatomical structure or structures whose constituents are metabolically inert is considerably supported by these findings. Particularly when taken in conjunction with similar results from the use of [4-^{14}C]cholesterol and [3-^{14}C]serine, it is reasonable to suppose that the inertness is due less to the molecular structure and biochemical properties of the constituents than to their presence in a stable anatomical unit.

SUMMARY

1. The fate of injected ^{32}P has been followed in blood and in the phospholipids of brain, spinal cord and sciatic nerve of developing rats for a period of 189 days after injection.

2. The fate of injected ^{32}P has been similarly followed in mature rats up to 179 days after injection.

3. Brain-lipid metabolism in developing and mature rats was also studied by means of [1-^{14}C]-glycerol.

4. A proportion of the phospholipids of rat nervous tissue appears to be metabolically inert.

The authors wish to thank Miss Jennifer Vogt for her valuable technical assistance.

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Biochem. J. (1960) **75**, 571

Phospholipid Metabolism in Nervous Tissue

3. THE ANATOMICAL DISTRIBUTION OF METABOLICALLY INERT PHOSPHOLIPID IN THE CENTRAL NERVOUS SYSTEM

BY A. N. DAVISON AND J. DOBBING
Department of Pathology, Guy's Hospital Medical School, London, S.E. 1

(*Received* 17 *August* 1959)

The metabolic inertness of a proportion of the phospholipids of nervous tissue has been demonstrated in earlier experiments by incorporating ^{32}P and ^{14}C into the brain, spinal cord and peripheral nerve of animals during early development, and by following the fate of the labelled material for periods from 6 months to a year after injection (Davison & Dobbing, 1960; Davison, Morgan, Wajda & Wright, 1959). It was suggested that this persistence was not due to any general metabolic inertness of particular cerebral phosphatides such as sphingomyelin, but rather that their metabolic stability was associated with their presence in certain anatomical structures, predominantly in the myelin sheaths. It follows from this hypothesis that it should be possible to incorporate labelled substances into the lipids of the myelin sheath only during the period of myelinogenesis. On the other hand, in adult animals after injection of ^{32}P virtually all the labelled phospholipid should undergo turnover.

In the preceding paper (Davison & Dobbing, 1960) the fate of ^{32}P in the phospholipids of the nervous system has been compared in developing and adult rats. It was found that a disproportionate persistence of acid-soluble ^{32}P in the adult blood compared with that in the young made the comparison of cerebral-phosphatide metabolism difficult. It was therefore decided to approach the problem from an anatomical standpoint.

Although the white matter of the brain contains as many cells as grey matter, much of the central-nervous-system myelin is located in the white matter. If the uptake of ^{32}P into white- and grey-matter phospholipid is expressed as specific activity, then in adults the white-matter specific activity should be lowered by the presence there of the inert proportion representing the fully developed myelin sheaths, and any isotope incorporated into phospholipids should not predominate in the more myelinated areas. Conversely, if the present hypothesis be true, that part of the phospholipid ^{32}P which persists in the brain after injection into developing animals should be located predominantly in the white matter. In this way it should be possible to detect a different anatomical pattern of distribution of phospholipid ^{32}P in the two age groups several weeks after injection of ^{32}P, while the radioactivity is still easily measurable. If metabolically inert phospholipid were found to be mainly situated in white matter, this would be a result similar to that previously obtained with [4-^{14}C]cholesterol (Davison, Dobbing, Morgan & Wright, 1959) and would be further presumptive evidence in favour of a metabolically inert myelin sheath.

METHODS

Two groups of rabbits of the same strain (Copenhagen White) were injected with ^{32}P. The first group consisted of 11 animals from two litters, each injected intraperitoneally when 16 days old with 250 μc of ^{32}P as phosphate. This represented a dose of about 1 μc of ^{32}P/g. body wt. at the time of injection. The second group of six adult rabbits, of 3 kg. average weight, each received intravenous injections of 500 μc of ^{32}P. Animals were killed singly with intravenous pentobarbitone, up to 180 days after injection, except for the final point in the developing series when two animals were killed together and their tissues were combined.

Brains were removed immediately after death and placed in formalin–0·9% sodium chloride soln. (10:90, v/v) for fixation for 24 hr. They were then dissected by a procedure already described (Davison, Dobbing, Morgan & Wright, 1959). Briefly, this involved cutting the whole brain into coronal slices about 2 mm. thick and then dividing the whole of each slice grossly into 'white' and 'grey' parts. In the forebrain and cerebellum this presented little difficulty. Wherever grey and white matter is not easily distinguishable, for example in much of the brain stem, equal amounts were allocated to the 'white' and 'grey' specimens. The result is a predominantly white and a predominantly grey specimen, each inevitably contaminated by the other, the two comprising the whole brain when added together.

Lipid extraction and counting procedure. The methods for the extraction of phospholipid and for the estimation of phosphorus were as previously described (Davison & Dobbing, 1959, 1960). Radioactivity was determined by liquid β counting up to 85 days after injection and by scintillation counting thereafter as reported in the preceding paper (Davison & Dobbing, 1960).

RESULTS

The method of dissecting the brains produced reasonably uniform samples, as can be seen from Table 1, in which the white fraction is expressed as a percentage of the whole brain. In the developing series white matter forms an increasing proportion of the wet weight and its phospholipid phosphorus content also rises during the first 100 days (the period during which myelin is being laid down).

The distribution of phospholipid ^{32}P between white and grey fractions up to 180 days after injection into adult animals is shown in Table 2. The specific activity in the white fraction is below or equal to that in the grey at all times except at the 180-days point, where it shows a slight excess. Towards the end of this period, however, the low radioactivity of the phosphorus increases the experimental error, and the difference between white and grey fractions is probably not significant.

The fate of phospholipid ^{32}P in 'white' and 'grey' fractions of brain in animals injected with inorganic ^{32}P when 16 days old is also shown in Table 2. With these animals, the specific activities have been individually corrected for the increments in phospholipid phosphorus occurring during growth, these levels in the 17-day-old rabbit being used as a reference point. As might be expected, phospholipid phosphorus increased in both fractions but disproportionately more so in the white matter (Table 1). Failure to make such corrections for growth would result in an apparent depression of the later phospholipid radioactivity and a spurious suggestion of loss, particularly in the white fraction. Table 2 shows that in about the first 40–50 days after injection, a turnover of phospholipid ^{32}P is taking place more in the grey fraction than in the white. Subsequently the decline in radioactivity up to 174 days after injection takes place almost wholly in the grey matter—that is, the white matter shows much greater persistence.

This relatively greater persistence of phospholipid-^{32}P radioactivity in the white fraction of the younger series is best shown by expressing the results as the ratios of white-matter to grey-matter specific radioactivity. In this way variability of dosage, concentrations of ^{32}P in the blood and biological variation between animals are eliminated. Fig. 1 shows that this ratio remains virtually at unity in the animals injected during adult life. In

Table 1. *Changes in weight and phospholipid-phosphorus content of 'white' matter in developing and adult rabbits*

White-matter fraction is expressed as a percentage of whole brain in young and old rabbits when dissected according to the method described in the text.

Age (days)	Time after injection (days)	Wet wt. of 'white' matter as % of whole brain	Phospholipid P in 'white' matter as % of total brain-phospholipid P
		Developing animals	
17	1	33	41
23	6	29	34
30	14	33	43
46	30	35	37
67	51	33	45
100	84	48	56
101	85	49	51
149	133	45	55
182	166	47	56
190	174	43	57
		Adult animals	
	7	55	56
	14	52	50
	23	57	63
	51	56	57
	85	47	66
	180	47	56

Table 2. *Specific activity of [^{32}P]phospholipid in 'white' and 'grey' matter in developing and adult rabbits*

Specific activity (counts/min./mg. of P) has been determined as described in the text. Results for developing rabbits have been corrected for phosphorus increments during growth. Figures for 174 days after injection are the means of two animals.

Time after injection (days)	Specific activity in rabbits injected with ^{32}P when 16-days old		Time after injection (days)	Specific activity in adult rabbits injected with ^{32}P	
	'White' matter	'Grey' matter		'White' matter	'Grey' matter
1	2 610	2 640	—	—	—
6	7 725	10 400	7	992	1310
14	9 930	10 500	14	1510	2240
30	13 150	16 300	23	2090	2480
51	9 930	7 220	51	1870	2190
84	12 560	5 810	—	—	—
85	11 200	7 040	85	1015	1035
133	7 830	4 080	—	—	—
166	7 450	2 290	—	—	—
174	8 800	2 110	180	1200	1060

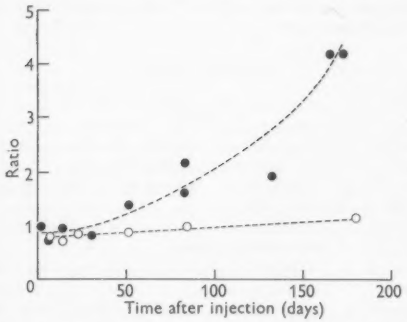

Fig. 1. Ratio of 'white'- to 'grey'-matter specific activity. Specific activities were determined by methods described in the text. Figures for 'white' matter from young rabbits have been corrected for increments of P during growth as reported in the text. Results show the ratios for young (●) and adult (○) rabbits.

those injected neonatally, the white : grey ratio increases from about 50 days after injection until, at 174 days the corrected specific radioactivity of phospholipid phosphorus in the white matter is four times that in the grey.

DISCUSSION

It is known that different phospholipids 'turnover' at widely different rates in the brain (Ansell & Dohmen, 1957), and also that any particular phospholipid may have different apparent rates of metabolism in animals of different ages (Ansell & Spanner, 1959; Miani & Bucciante, 1957). It seems equally probable that in an organ so complex structurally as the brain, the rate of metabolism of such substances may depend as much on their location in tissue elements as on their chemical constitution. This heterogeneity takes an extreme form when the substance is metabolically inert in some components of the organ while subject to turnover in others, as might happen were it present in myelin or in cells. During the growth and maturation of the central nervous system, the position is further complicated by the fact that the spectrum of turnover rates for particular phospholipids may change with increase in the age of the animal. Any valid comparison of the phospholipid metabolism of animals at different stages of development thus presents a number of difficulties, only some of which have been solved.

When labelled materials are incorporated into the brain during development, their persistence in any metabolically inert structure may be seriously underestimated if results are expressed as specific activities, or in any other way as concentrations, since these labelled compounds are progressively diluted by unlabelled compounds taken up during normal growth. To obviate this source of error, our results have previously been expressed in terms of whole organs.

If it is desired to study relative persistence in the various gross anatomical divisions of the brain, results could be expressed by taking whole cerebrum, whole cerebellum, etc. (see Fries & Chaikoff, 1941*a*, *b*). On the other hand, any investigation of metabolism or persistence in a microscopical structural component in the tissues, such as the myelin sheath, which is common to all the major anatomical divisions of the central nervous system, is at present possible only by dissecting the whole brain as nearly as possible into its 'white' and 'grey' matters. This unfortunately makes it impossible to express results in terms of 'whole structure', and necessitates the use of specific-activity-correction devices to compensate for the effects of growth. The method of compensation employed here for the series of immature rabbits has been to correct each phospholipid-phosphorus specific-activity value for growth increments of phospholipid phosphorus, using the first 17-day-old animal as an arbitrary reference point. This correction factor, which is based on brain-phospholipid phosphorus at various ages, never exceeds 3·5. This procedure introduces several theoretical objections, the chief of them being the implied assumption that subsequent growth increments have not added to the radioactivity of those extracted phospholipids by further incorporation of additional labelled precursor. However, in the post-injection period, the levels of circulating precursor ^{32}P fall more rapidly in developing than in mature animals so that errors from this source are unlikely (Davison & Dobbing, 1960). Moreover, the present investigation is principally concerned with the location of the persisting phospholipid ^{32}P long after the blood levels in both immature and adult rabbits have become insignificant.

From this present study on phospholipid metabolism in the brain, two facts seem to emerge. First that [^{32}P]phospholipid persists in the white matter and not in the grey matter after injection of ^{32}P into young rabbits. This finding confirms earlier work (Davison, Morgan, Wajda & Wright, 1959) with [3-^{14}C]serine in which it was demonstrated that sphingomyelin and cephalins undergo little turnover in the brain up to 250 days after injection. In that paper it was also shown that in an animal killed after 250 days, four times as much radioactive sphingomyelin and cephalin was present in the 'white' as in the 'grey' matter. Secondly, a comparison of the ratio of radioactivity in 'white' and 'grey' matter obviates many errors and Fig. 1 shows how large are the differences that develop

with time in this ratio for the metabolism of ^{32}P when injected into developing or mature rabbits. This work is therefore fully consistent with the hypothesis that the lipids of the myelin sheath are metabolically inert. Furthermore, it ought to be possible, by more refined anatomical and biochemical techniques, to establish the hypothesis in even greater detail.

SUMMARY

1. ^{32}P has been injected into developing and adult rabbits and its fate investigated in the phospholipids of white and grey matter of brain.

2. Those phospholipids which have been found to persist are predominantly located in the white matter.

Our thanks are due to Miss Jennifer Vogt for her able technical assistance.

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Metabolism of Myosin and Life Time of Myofibrils

BY J. C. DREYFUS, J. KRUH AND G. SCHAPIRA
Laboratoire de Recherches de Biochimie Médicale, Hôpital des Enfants-Malades, 149 rue de Sèvres, Paris 15e, France

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The concept of the dynamic state of body proteins is now generally admitted, but recent work has made some reappraisal necessary. Proteins may undergo three types of metabolic patterns. Some turn over at a rather fast rate, but the turnover is the result of a secretion out of the cells: such is true of digestive enzymes or of the plasma proteins as studied by Miller, Bly, Watson & Bayle (1951). Others remain in the cells and undergo turnover; Dreyfus, Schapira & Kruh (1956) have shown that this is so with muscle aldolase. On the other hand Shemin & Rittenberg (1945) demonstrated that haemoglobin does not turn over during the life of the cell. Later this concept was applied by Neuberger, Perrone & Slack (1951) and by Robertson (1952) to collagen, which might remain inert during the whole life of the animal. Thompson & Ballou (1956) have emphasized the quantitative importance of apparently inert proteins. Shemin & Rittenberg (1944) showed that the muscle proteins can be divided into two groups, one of which is rapidly metabolized and the other slowly metabolized. Myosin belongs to the slowly metabolized group (Bidinost, 1951).

Neuberger (1952) suggested that 'the metabolic activity of actin or myosin in the muscle fibre, or that of collagen, for example, is not a continuous function, but varies in a discontinuous manner and is dependent on morphological changes of the structures with which these proteins are associated'. This idea has never been experimentally checked for muscle proteins. In this work we have tried to determine whether myosin is in a dynamic equilibrium or whether it is inert enough to permit the determination of the life span of the cell or of a part of the cell. A preliminary note has been previously published (Dreyfus, Kruh & Schapira, 1957).

METHODS

Injection of radioactive glycine. White male rats of pure Wistar strain, weighing 200–250 g., fed with an identical diet, were used in each series of experiments. In these conditions, the specific activity of radioactive proteins from two animals given the same diet did not differ by more than 15%. The animals were given two types of diet, a 'standard' diet and a 'protein-rich' diet. The standard diet was a commercial one containing (%): oats (3), corn (5), wheat (30), molasses (2), wheat and barley seeds (8), lucerne flour (4), defatted peanut meal (5), defatted soya meal (11), defatted flax meal (5), skimmed-milk powder (3), meat flour (4), fish flour (4) and brewer's yeast (3), together with salts and vitamins. This standard diet contained approx. 25% of protein. The 'protein-rich' diet was pre-

pared by mixing 73 parts of the standard diet with 27 parts of casein, and it contained 45% of protein. The animals were starved for 16 hr. before and 6 hr. after the injection of radioactive glycine. Glycine uniformly labelled with ^{14}C was injected intraperitoneally, at a dosage of 5 μc (100 μg.)/100 g. body wt. In one experiment [^{14}C]valine was used. The animals were killed by bleeding after chloral anaesthesia; tissues were immediately excised and put in ice.

Muscle proteins. Muscles were crushed in a Latapie's apparatus. About 30 g. was obtained from each rat. Myosin was prepared according to Szent-Gyorgyi (1951); its purity was ascertained by moving-boundary electrophoresis. The absence of actomyosin was always checked by the absence of fall in viscosity under the action of adenosine triphosphate. Myosin was precipitated by 20% trichloroacetic acid. Aldolase was crystallized three times by the method of Warburg & Christian (1943) modified by Taylor, Green & Cori (1948). The purity of the protein was verified in preliminary experiments by electrophoresis and by the absence of activity of phosphorylase, phosphoglucomutase, phosphoglyceraldehyde dehydrogenase and α-glycerophosphate dehydrogenase. Water-soluble proteins were prepared by extraction of crushed muscle with 4 vol. of water and, after filtration, by precipitation of the protein with 1 vol. of 20% trichloroacetic acid.

Myofibrils. Myofibrils were prepared according to Perry (1953). Muscles were crushed with Latapie's apparatus and then homogenized in a Waring Blendor in borate buffer during 90 sec. in the cold room. Myofibrils were isolated and purified by five centrifugings at 600 *g* for 20 min., the upper part of the residue being collected each time, then by centrifuging at 300 *g* for 2 min., at which speed impurities are deposited. Microscopic examinations were made and the proteins were precipitated by 1 vol. of 20% trichloroacetic acid, then dissolved again in N-NaOH and reprecipitated by 20% trichloroacetic acid.

Globin. The red cells were washed three times in 0·9% NaCl, lysed by addition of 1 vol. of water, shaken manually with 0·4 vol. of toluene for 5 min. and allowed to stand overnight at 4°. After centrifuging, the haemoglobin layer was removed, globin was precipitated and separated from haemin by addition of acetone containing 1% of HCl according to Anson & Mirsky (1930); the haemin was removed by filtration and globin was washed with acetone and ether.

Isolation of glycine from proteins. Proteins were hydrolysed by heating under reflux with 3000 vol. of 6N-HCl for 48 hr. at 112°. Hydrochloric acid was eliminated and the residue was dissolved in water and evaporated to dryness *in vacuo* six times. Glycine was isolated as dinitrophenylglycine (DNP-glycine), after treatment of the hydrolysate by dinitrofluorobenzene, on a Celite column by a modification by Kruh, Dreyfus, Schapira & Padieu (1957) of Perrone's (1951) method. The purity of DNP-glycine was determined by a two-dimensional paper chromatography according to Levy (1954), by determination of the melting point of the compound and of its mixture with the synthetic compound. The DNP-glycine was dissolved in methanol.

Isolation of free glycine. Trichloroacetic acid was removed with ether and the solution was treated with dinitrofluorobenzene; the DNP-glycine was isolated and its purity determined as described above.

Determination of radioactivity. The proteins were washed twice with 5% trichloroacetic acid, twice with acetone and once with ether. They were crushed into a fine powder in an agate mortar and spread in layers of 'infinite thickness' on a copper planchet (2·85 cm.²). The specific activity of DNP-glycine is expressed in counts/min./m-mole. Its concentration was measured with a spectrophotometer at 360 mμ, after dilution with 2% $NaHCO_3$; its radioactivity was determined by drying another portion in a copper planchet in which a thin paper had been fixed with silicone. Determinations were carried out with a standard error of less than $\pm 2\%$; corrections for thickness were made.

RESULTS

The incorporation of glycine into myosin is maximum by 36 hr.; the radioactivity of the myosin remains constant until about the thirtieth day and then decreases to a lower plateau, and the radioactivity of aldolase decreases constantly in animals fed with a diet containing 24% of protein. Table 1 shows a typical result obtained in one series of animals; Fig. 1 shows the results obtained with all the rats. The curve of the specific activity of glycine isolated from myosin (Fig. 2) is similar, and the specific activity of free glycine in muscle decreases exponentially.

Nevertheless, the spread of the experimental points requires other evidence for this interpretation

Table 1. *Radioactivity of myosin and aldolase after injection of* [^{14}C]*glycine into rats fed with the standard diet* (24% *of protein*)

One series of rats was used.

Days after injection	Radioactivity (counts/min.) Myosin	Aldolase	Ratio: Aldolase / Myosin
2	41	134	3·3
5	38	112	2·95
11	37	100	2·7
15	47	81	1·7
23	40	70	1·7
26	48	65	1·35
39	20	43	2·15

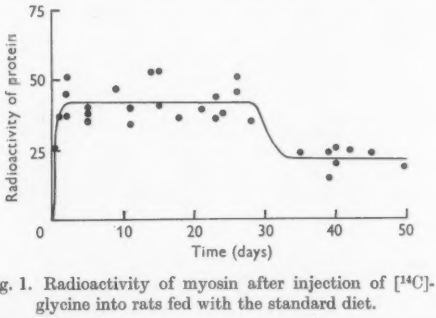

Fig. 1. Radioactivity of myosin after injection of [^{14}C]-glycine into rats fed with the standard diet.

of the shape of the radioactivity curve of myosin. The plateau is ascertained by the constant ratio of the radioactivity of myosin to globin between the fifth and the thirtieth days (Fig. 3); this supports an identical behaviour of myosin and globin during this period (later there seems to be a temporary decrease due to the drop of the radioactivity of myosin). The initial decrease of the ratio is due to the lag in the appearance of circulating radioactive red cells: the specific activity of haemoglobin increases for a week, whereas that of myosin reaches its maximum within 2 days after the injection. This decrease in the radioactivity of the myosin is further suggested by the shape of the curve of the ratio of the radioactivity of aldolase or soluble protein of muscle to that of myosin. Fig. 4 shows that the ratio curve is descending first, due to the decrease of the radioactivity of aldolase (Fig. 4*A*) and of the soluble proteins of muscle (Fig. 4*B*); after 32 days, the slope of the curve is reversed, which can be explained by the sudden fall of the radioactivity of the myosin. After 40 days the curve decreases again, since myosin has reached a new plateau. A similar result was obtained in one series in which [^{14}C]valine was used instead of [^{14}C]glycine (Fig. 4*C*).

The ratio of radioactivity (total protein of myofibrils/myosin), which is 1·21 at the third day, approaches 1 and remains so until the end of the experiment (42 days) (Table 2).

High-protein diet. In rats given a protein-rich diet, the curve has a different shape and does not show a plateau. Table 3 gives the result of one typical experiment. Fig. 5 gives the result for all the animals. The dispersion of the results makes it difficult to draw a curve, but it can be seen that the curve would decrease slowly without displaying any apparent fall. Nevertheless, the ratio of the radioactivity of the soluble protein of muscle to

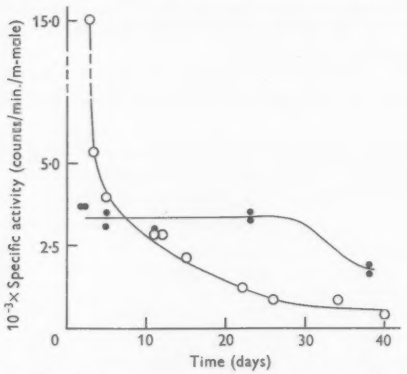

Fig. 2. Specific activities of glycine in myosin (●) and free glycine (○) of muscle (measured as DNP-glycine) in rats fed with the standard diet, after injection of [^{14}C]-glycine.

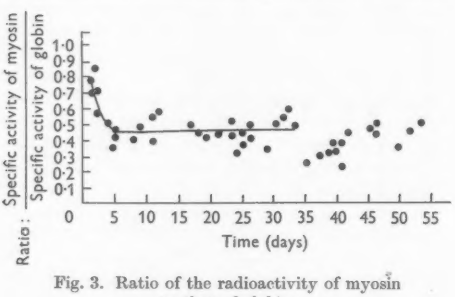

Fig. 3. Ratio of the radioactivity of myosin to that of globin.

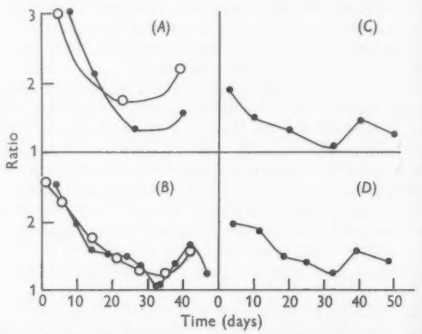

Fig. 4. Ratio of radioactivities of muscle proteins. (*A*) Aldolase/myosin, after injection of [^{14}C]glycine into rats fed on the diet with 24% of protein (two different series of animals were used). (*B*) Muscle soluble protein/myosin in the same conditions (two different series of animals were used). (*C*) Muscle soluble protein/myosin after injection of [^{14}C]valine into rats fed on the diet with 24% of protein. (*D*) Muscle soluble protein/myosin after injection of [^{14}C]glycine into rats fed on the diet with 45% of protein.

Table 2. *Ratio of the radioactivity of total myofibril proteins to that of myosin after injection of* [^{14}C]*glycine*

Rats were fed with a diet containing 24% of protein.

Days after injection	Ratio of radioactivities: myofibril proteins / myosin
3	1·21
9	1·15
14	1·11
24	1·02
28	1·00
35	1·00
42	1·00

Table 3. *Radioactivities of myosin and soluble proteins after injection of* [^{14}C]*glycine into rats fed with the high-protein diet* (45% *of protein*)

One series of rats was used.

Days after injection	Radioactivity (counts/min.) Myosin	Radioactivity (counts/min.) Soluble proteins of muscle	Ratio of the radioactivities: soluble proteins / myosin
4	57	115	2·0
11	63	115	1·85
18	46	69	1·50
25	35	51	1·45
32	45	56	1·25
39	30	48	1·60
48	20	29	1·45
53	25	34	1·35

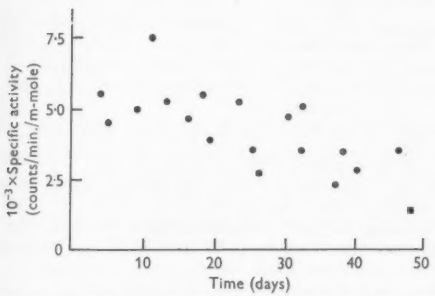

Fig. 5. Specific activity of glycine in myosin (measured as DNP-glycine) after injection of [^{14}C]glycine into rats fed with the high-protein diet.

that of myosin (Fig. 4*D*) presents the same reversal of the slope as that with rats fed with the standard diet.

DISCUSSION

Myosin and life time of myofibrils. The nearly constant radioactivity of myosin (Fig. 1) can be interpreted to mean, as with haemoglobin, that myosin is not in a dynamic equilibrium. In the same way as the behaviour of haemoglobin permits one to determine the life span of the red cells, the behaviour of myosin may permit one to measure the life span of the muscle cell or, more likely, of a sub-unit of the cell.

Recently Leblond & Walker (1956) have discussed the notion of cellular renewal in a large number of cell populations, the cells of the haemopoietic tissues and the red cells being the best-investigated types. Among fixed tissues, the epithelial cells are continuously renewed; on the other hand, the duration of life of hepatic cells has been estimated to be at least 4–5 months (Swick, Koch & Handa, 1956); that of the muscle cells has not been investigated so far. Their life duration seems to be very long in view of the absence of mitotic activity; the rate of turnover of the various cellular sub-units varies widely and we can interpret our findings in the light of the life span of a subcellular unit likely to be the myofibril. This notion has been considered by several histologists. Whereas the division of the myofibrils has been observed only very rarely, by Adams, Denny-Brown & Pearson (1954), it has been noted that the phenomena of muscle hypertrophy or atrophy was accompanied by variation in the number of the myofibrils. In our experiments the plateau is reached after 2 days and the decline in radioactivity of the myosin takes place between 28 and 35 days. The molecules of myosin are therefore destroyed about 30 days after being synthesized. Since the radioactivity of myosin is very similar to that of the total myofibril proteins, it seems that the curve of the radioactivity of myosin reflects the life span of myofibrils. The decline in over-all radioactivity of the myofibril protein, compared with that of myosin, can be explained by the fact that the myofibril contains a small amount of protein with a fairly rapid turnover, but the bulk of its protein is composed of myosin and perhaps other proteins with an apparent metabolic inertness and the same radioactivity as myosin. These results permit us to ascribe to the myofibrils of the rat a life span of 30 days (unpublished experiments have shown a life duration for mice myofibrils of 20 days). The values given are only average values for the whole animals, since the incorporation of the amino acid may vary with the site of the muscle, as has been shown previously with [^{15}N]-glycine by Schapira, Coursaget, Dreyfus & Schapira (1953). Variation of incorporation with the site has also been shown in epidermal and intestinal cells by Hooper (1956). However, our results demonstrate the possibility of determining the life span of a subcellular unit by biochemical methods.

Myosin and dynamic equilibrium. The experiments performed with rats fed with a diet containing 24% of protein led us to conclude that myosin is metabolically inert or almost inert. However, this inertness may only be apparent since free glycine of muscle remains significantly radioactive for several weeks, as we have shown previously (Schapira, Dreyfus, Kruh, Labie & Padieu, 1958). This radioactive precursor should tend to increase the specific activity of myosin, even after the first days: if myosin was completely inert for 30 days, the specific activity of free glycine would be enough to increase the over-all activity of myosin, since at that time only non-radioactive myosin would be destroyed. Since we observed a plateau and no increase, it is probable that either some molecules of radioactive myosin are destroyed

during the life of the myofibril (compensating for the new molecules of radioactive myosin which are being synthesized) or that there is some turnover of the myosin molecule which decreases progressively its radioactivity. The plateau would, in this case, represent the net result of the two mechanisms. To demonstrate this we decided to feed rats with a diet enriched in protein, in order to dilute as fast as possible the free radioactive glycine.

Steinbock & Tarver (1954) have shown that a protein-rich diet apparently accelerated the renewal of proteins, a possible consequence of the decrease in re-utilization of the radioactive amino acid. In the experiments with a protein-rich diet we observed a disappearance of the plateau and the radioactivity progressively decreased, with a large dispersion of the points. It appears, under these conditions, that a molecular renewal of myosin is demonstrated. It is impossible to demonstrate from the curves the life duration of myofibrils: our conclusion is, however, supported by the fact that by about the thirtieth day, the slope of the curve of radioactivities (water-soluble protein/myosin) is reversed (Fig. 4*D*).

We can therefore come to the following conclusions: the myofibrils display a definite life span of about 30 days; the myosin is in a state of relative inertness and its metabolic behaviour can be compared with that of haemoglobin; the inertness, however, is not complete; if the radioactive free glycine is diluted by the use of a high-protein diet, the myosin molecule appears to have a slow but definite molecular turnover.

SUMMARY

1. After the injection of radioactive glycine into groups of rats on a diet containing 24% of protein, myosin was isolated at various times.

2. The specific activity of the myosin (and of the glycine of the myosin) remained constant for about 30 days and then decreased.

3. The ratio of the specific activity of the myosin to that of erythrocyte globin was constant between about 5 and 30 days after injection.

4. On this basis the life span of the myofibril is considered to be 30 days on average.

5. When the animals received a diet containing 45% of protein the radioactivity of myosin progressively decreased, showing that the protein undergoes turnover.

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The Enzymic Activation of D-Alanine

By J. BADDILEY and F. C. NEUHAUS*
Chemistry Department, King's College, Newcastle upon Tyne, 1

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D-Alanine is a major component in cell-wall preparations obtained from a variety of micro-organisms (Snell, Radin & Ikawa, 1955; Salton, 1957). In addition, its presence has been demonstrated in intracellular nucleotides (Park, 1952*b*; Strominger, Scott & Threnn, 1959) which accumulate when *Staphylococcus aureus* and *Escherichia coli* are treated with penicillin. The nucleotides are believed to be precursors of wall material of these bacteria (Park & Strominger, 1957; Strominger & Threnn, 1959). Moreover, D-alanine is required for growth of several lactic acid bacteria in media deficient in vitamin B_6 (Snell, 1945; Holden & Snell, 1949).

Snell *et al.* (1955) have shown that D-alanine is present 'in two distinct forms' in the walls of *Streptococcus faecalis* and various lactic acid bacteria. In *S. faecalis* 40 % of the D-alanine of the walls can be extracted with hot trichloroacetic acid as a bound form which is described as 'especially labile to alkaline hydrolysis', whereas the remaining D-alanine, which is insoluble in trichloroacetic acid, appears to be bound as a 'protein-like material'. The similarity in properties and method of extraction of the former fraction suggests that these authors had obtained preparations containing teichoic acids (Armstrong, Baddiley, Buchanan & Carss, 1958; Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958), which are present in the walls of a variety of micro-organisms including those of *S. faecalis* (Armstrong, Baddiley, Buchanan, Davison, Kelemen & Neuhaus, 1959). These teichoic acids contain alanine ester groups which are labile to neutral hydroxylamine, and in the compounds from *Staphylococcus aureus* H and *Lactobacillus arabinosus* the alanine has the D-configuration. Recent investigations by Ikawa & Snell (1958) have demonstrated the presence of D-alanyl-D-alanine as a major product of D-alanine metabolism in *Streptococcus faecalis*.

The wide distribution of D-alanine in a variety of bound forms in bacteria has directed attention to its metabolism in cell-free extracts. This report describes the presence, partial purification and characteristics of an amino acid-activating enzyme for D-alanine from *L. arabinosus* 17–5. A preliminary report of this work has been presented (Baddiley & Neuhaus, 1959). In contrast with other amino acid-activating enzymes, this is the first example of an enzyme for activating a D-amino acid. The process for the activation of D-alanine is analogous to that for the L-amino acids, which was first discovered by Hoagland, Keller & Zamecnik (1956) and demonstrated later with more highly purified enzymes (Davie, Koningsberger & Lipmann, 1956; Berg, 1956; DeMoss, Genuth & Novelli, 1956; Holley & Goldstein, 1959; van de Ven, Koningsberger & Overbeek, 1958; Schweet & Allen, 1958; Webster & Davie, 1959; Bergmann, Berg, Preiss, Ofengand & Dieckmann, 1959). According to currently accepted views, the activation step would be represented by reaction (1):

$$\text{Enzyme} + \text{D-alanine} + \text{ATP} \xrightleftharpoons{Mg^{2+}\ \text{ion}} \text{Enzyme–AMP–D-alanine} + \text{pyrophosphate}. \quad (1)$$

Enzymes of this type catalyse the formation of amino acid hydroxamates from adenosine triphosphate (ATP), hydroxylamine and the amino acid in the presence of Mg^{2+} ions (AMP, adenosine monophosphate). Moreover, with only one exception (Cormier & Novelli, 1958), these enzymes catalyse an amino acid-dependent exchange of inorganic [^{32}P]pyrophosphate into ATP. The above reactions are represented for the D-alanine-activating enzyme as follows:

$$\text{ATP} + \text{D-alanine} + \text{hydroxylamine} \xrightarrow{Mg^{2+}\ \text{ion}} \text{D-alanine hydroxamate} + \text{AMP} + \text{pyrophosphate} \quad (2)$$

$$\text{ATP} + [^{32}\text{P}]\text{pyrophosphate} \underset{\text{(D-alanine)}}{\rightleftharpoons} [^{32}\text{P}]\text{ATP} + \text{pyrophosphate}. \quad (3)$$

Amino acid-activating enzymes for L-amino acids have been found in micro-organisms (DeMoss & Novelli, 1956; Nismann, Bergmann & Berg, 1957; Novelli, 1958; Schweet, Holley & Allen, 1957),

* Postdoctoral Fellow of the National Science Foundation (U.S.A.). Present address: Biochemistry Division, Department of Chemistry, University of Illinois, Urbana, Ill., U.S.A.

animal tissues (Schweet *et al.* 1957; Hoagland *et al.* 1956; Davie *et al.* 1956; Lipmann, 1958) and plant material (Davis & Novelli, 1958; Marcus, 1959; Clark, 1958; Webster, 1957).

EXPERIMENTAL

Materials

D-Alanine, D-α-amino-*n*-butyric acid and D-serine were purchased from the California Corp. for Biochemical Research. Crystalline ATP and adenosine diphosphate (ADP) were the products of Pabst Laboratories and the triphosphates of cytidine, uridine and guanosine were purchased from Sigma Chemical Co. [^{32}P]Pyrophosphate containing less than 0·5% of inorganic phosphate was obtained from The Radiochemical Centre, Amersham, Bucks.

Calcium phosphate (hydroxylapatite modification) was prepared according to the method of Tiselius, Hjertén & Levin (1956). DL-Alanine hydroxamic acid was synthesized by the method of Safir & Williams (1952) and used as a colorimetric standard in the hydroxamate assay. Salt-free hydroxylamine was prepared by the method of Beinert *et. al.* (1953), and its concentration was determined colorimetrically (Frear & Burrell, 1955). Yeast inorganic pyrophosphatase was prepared by the 'Alternative purification procedure' of Heppel (1955) through the ethanol fractionation. The specific activity of this preparation was 11 200 μmoles/hr./mg. of protein.

Analytical procedures

In enzyme preparations that had not been treated with protamine sulphate, the protein concentration was determined with the Folin reagent (Lowry, Rosebrough, Farr & Randall, 1951) with bovine serum albumin as a standard. In preparations containing less than 8% of nucleic acid, protein was determined by the spectrophotometric method (Warburg & Christian, 1941). Good agreement was observed between the two methods. Orthophosphate was determined by the method of Fiske & Subbarow (1925). Radioactivity measurements were made with a thin-window Geiger–Müller counter on polyethylene planchets (1·77 cm.^{2}) to which 0·05 ml. of 0·01 M-cetyltrimethylammonium bromide had been added. All radioactivity measurements were made with a counting error of less than 5%.

Assay procedures

Hydroxamate assay. The complete system contained: 10 μmoles of $MgCl_2$; 100 μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 7·4; 800 μmoles of salt-free hydroxylamine, pH 7·4; enzyme preparation; 9 μmoles of neutralized ATP; 100 μmoles of D-alanine; water to a final volume of 1 ml. Unless otherwise stated the assay solutions contained 40 μg. of purified inorganic pyrophosphatase/ml. The tubes were incubated for the indicated period at 37°. The reaction was stopped and the colour developed by the method of Cormier & Novelli (1958). In every case control experiments were performed without added D-alanine to eliminate the effects of chromogenic impurities and endogenous activity due to the presence of other amino acids.

A unit of enzyme is defined according to various authors (van de Ven *et al.* 1958; Davie *et al.* 1956; Schweet & Allen, 1958) as the amount of enzyme which catalyses the formation of 1 μmole of hydroxamate/hr. Specific activity is expressed as the number of units/mg. of protein.

The proportionality of the hydroxamate assay is shown in Fig. 1. Fig. 2 (curve *B*) demonstrates that the rate of hydroxamate formation is linear for 1 hr. All assays for hydroxamate formation were measured under conditions in which linearity was observed.

Exchange assay. The complete system for measuring the [^{32}P]pyrophosphate–ATP-exchange reaction contained: 10 μmoles of $MgCl_2$; 50 μmoles of tris–HCl buffer, pH 7·8;

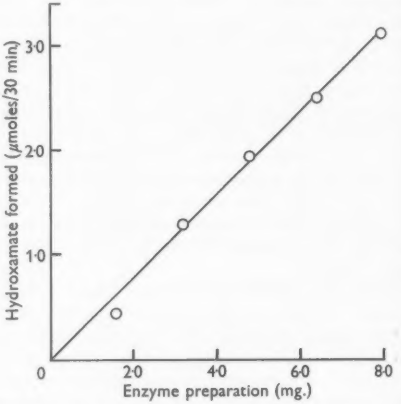

Fig. 1. Effect of enzyme concentration on hydroxamate formation. The purified preparation through step 3 was used in the hydroxamate assay.

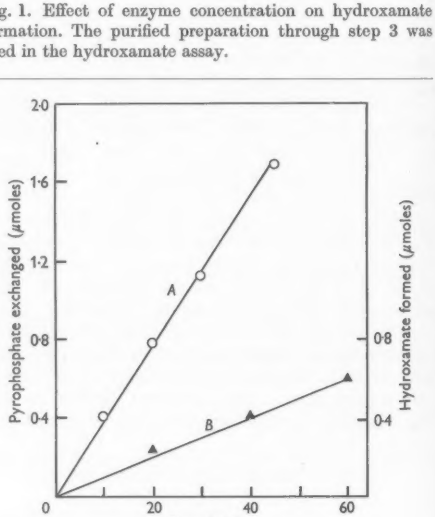

Fig. 2. Exchange of [^{32}P]pyrophosphate (curve *A*) and hydroxamate formation (curve *B*) as a function of time. The exchange assay was performed with 20 μg. of purified enzyme preparation and the hydroxamate assay was carried out with 23 μg. of protein.

enzyme preparation; 9 μmoles of dipotassium ATP neutralized with NaOH; 7 μmoles of [^{32}P]pyrophosphate (average specific activity 50 000 counts/min./μmole); 100 μmoles of D-alanine; water to a final volume of 1 ml. Unless otherwise stated the tubes were incubated at 37° for 30 min., and the reaction was terminated by the addition of 1 ml. of 10% trichloroacetic acid. ATP and pyrophosphate were separated by the method of Crane & Lipmann (1953). After acid hydrolysis of the ATP, radioactivity and orthophosphate were determined in measured portions. The amount of [^{32}P]pyrophosphate exchanged was calculated by the method of Duffield & Calvin (1946). Unless otherwise specified all values are corrected for a small amount of apparent non-enzymic [^{32}P]pyrophosphate exchange. Exchange results are reported as μmoles of [^{32}P]pyrophosphate exchanged/hr. or, in some cases, as % exchanged/30 min.

Fig. 2 (curve *A*) illustrates the effect of time on the [^{32}P]pyrophosphate–ATP-exchange reaction. Under the assay conditions employed the velocity of the [^{32}P]pyrophosphate–ATP exchange to hydroxamate formation is 4·5.

Growth of bacteria and preparation of extracts

Lactobacillus arabinosus 17·5. This was maintained on litmus–milk culture and was grown at 28° in the following medium: glucose, 2%; (autoclaved separately); tryptone, 2%; sodium acetate, 1%; yeast extract (Oxoid), 1%; potassium dihydrogen phosphate, 0·45%; N-sodium hydroxide, 26 ml./l.; inorganic salts 'B' (Barton-Wright, 1946), 5 ml./l. The cells were grown in the medium for 18 hr. and then harvested in a Sharples centrifuge. The wet cells were resuspended in 0·01 M-phosphate buffer, pH 6·8, and centrifuged again. Acetone-dried cells were prepared by suspending 40 g. of wet cells in 400 ml. of acetone (previously chilled to −20°) and homogenizing in a blender for 1 min. The mixture was filtered on a Büchner funnel through Whatman no. 1 filter paper, and the filter cake was suspended in 200 ml. of acetone at −20°. The suspension was filtered and approximately 200 ml. of peroxide-free ether at 20° was used to wash the cells on the funnel. The cells were then dried at room temperature, and last traces of ether were removed *in vacuo*. The dried cells were stored at −20°. The product from 220 g. of wet cells weighed 41 g.

To prepare the crude extract dried cells (9 g.) suspended in 75 ml. of 0·01 M-phosphate buffer, pH 6·8, were disrupted at 2° in three lots for 1 hr. with the aid of the 20 kcyc./sec., 60 w MSE–Mullard Ultrasonic Disintegrator (Measuring and Scientific Equipment Ltd.) with the 1:1 vibrator probe. Unbroken cells and debris were removed by centrifuging at 5° for 30 min. at 21 500 *g*.

Lactobacillus casei, Bacillus subtilis *and* Staphylococcus aureus *H. L. casei* ATCC 7469 (kindly provided by Dr E. F. Gale) was grown under the same conditions as *L. arabinosus*. A cell-free extract was prepared by disrupting 2 g. of acetone-dried cells in 20 ml. of 0·02 M-phosphate buffer, pH 6·8, for 30 min. Unbroken cells and debris were removed by centrifuging at 21 500 *g* at 2° for 30 min. *S. aureus* H (kindly provided by Dr J. T. Park) was grown overnight at 37° with aeration on a medium similar to that described by Park (1952*a*). A cell-free extract was prepared by disrupting 0·35 g. of acetone-dried cells in 5 ml. of 0·01 M-phosphate buffer, pH 6·8, for 25 min., and then centrifuging as described for *L. casei*. *B. subtilis* (kindly provided by Dr M. R. J. Salton) was grown overnight at 28° with aeration on the following medium: peptone, 2%; NaCl, 0·5%; DL-alanine, 0·4%. The DL-alanine was included in the medium in an effort to inhibit spore formation (Foster & Heiligman, 1949). Wet cells (4·5 g.) in 20 ml. of 0·01 M-phosphate buffer, pH 6·8, were disrupted for 25 min. and centrifuged as described before.

Purification of enzyme from extracts of Lactobacillus arabinosus

Ammonium sulphate precipitation and protamine sulphate treatment. All subsequent steps were carried out at 0–4°. The extract (50 ml.) was brought to 0·80 saturation by the slow addition of 26 g. of ammonium sulphate. When the solution had been stirred for 1 hr. the precipitate was removed by centrifuging for 20 min. at 21 500 *g* and dissolved in 0·01 M-phosphate buffer, pH 6·8. This fraction was dialysed in Visking cellophan tubing for 4 hr. against 0·01 M-phosphate buffer, pH 6·8. Protamine sulphate (1%), which had been adjusted to pH 5, was added dropwise to this fraction until the supernatant obtained by centrifuging had an absorption ratio at 280–260 mμ of 0·75. The precipitate was removed by centrifuging.

Ammonium sulphate fractionation. The supernatant (53 ml.) from the above step was brought to 0·55 saturation by the slow addition of 17·3 g. of ammonium sulphate. The precipitate was removed by centrifuging at 21 500 *g* and discarded. The supernatant was brought to 0·70 saturation by the addition of 4·9 g. of ammonium sulphate. After the mixture had been stirred for 15 min., the precipitate was collected by centrifuging at 21 500 *g* and dissolved in 0·01 M-phosphate buffer, pH 6·8. This fraction was dialysed against 4 l. of the same buffer for 4 hr.

Chromatography on calcium phosphate (hydroxylapatite modification). A column (13 cm. × 1 cm.) was packed with calcium phosphate under slight positive pressure and washed for 12 hr. with 0·01 M-phosphate buffer, pH 6·8. The enzyme solution (1 ml., 125 units) was applied to the column, which was then developed successively with 0·01, 0·03, 0·05, 0·10 and 0·20 M-phosphate buffer, pH 6·8. A flow rate of 0·6 ml./min. was maintained by a pressure system. The fractions (3 ml.) which contained the bulk of the D-alanine-activating enzyme were combined and stored at −10°. Fig. 3 illustrates a typical separation.

The original extract, the supernatant from the treatment with protamine sulphate and the ammonium sulphate (0·55–0·70) fraction were stable for 6 months at −10°. However, the fraction obtained from the column lost approximately 25% of its activity at −20° during 1 week. The specific activities reported for the fractions from the column refer to freshly prepared material. A summary of the purification procedure is presented in Table 2.

RESULTS

Distribution of activating enzyme in various bacteria

The results in Table 1 demonstrate that *L. casei* was the most abundant source of the D-alanine-activating enzyme. The presence of the enzyme has now been demonstrated in extracts of *Streptococcus faecalis* R (ATCC 8043). In every case activity towards L-alanine was either absent or very low.

Schweet *et al.* (1957) noted that histidine, methionine, serine and valine are activated in extracts of *Lactobacillus plantarum*. In addition to D- and L-alanine, these amino acids were also activated in the hydroxamate assay by unpurified enzyme preparations from *L. arabinosus*. The D-alanine-activating enzyme from *L. arabinosus* was chosen for purification because of its possible participation in the system which incorporates D-alanine into the teichoic acid of that micro-organism.

Properties of the purified enzyme

The requirements for the hydroxamate system and the [^{32}P]pyrophosphate–ATP-exchange reaction are given in Table 3. Both reactions show an absolute requirement for Mg^{2+} ions and D-alanine. The hydroxamic acid was identified as alanine hydroxamate by cochromatography with authentic material by the following procedure. A system containing 200 μmoles of D-alanine, 1600 μmoles of hydroxylamine (pH 7·4), 200 μmoles of tris–HCl buffer (pH 7·4), 20 μmoles of $MgCl_2$, 4·5 mg. of enzyme preparation purified through step 3, 18 μmoles of neutralized ATP and water to 2 ml. was incubated for 1 hr. at 37°. The reaction was terminated by placing the incubation mixture in a boiling-water bath for 3 min. After removal of the protein by centrifuging, the supernatant was evaporated to dryness in a desiccator over H_2SO_4 to decompose the hydroxylamine. The sample was partially deionized on Dowex-1 (OH^- form) according to the procedure of Stein (1953). The residue was chromatographed in the following ascending solvent systems: (*a*) butan-2-ol–formic acid–water (75:15:10, by vol.) (Hoagland *et al.* 1956); (*b*) butan-1-ol–acetic acid–water (4:1:5, by vol.) (organic layer); (*c*) ethanol–aq. NH_3 (sp.gr. 0·88)–water (10:1:2, by vol.) (Schweet *et al.* 1957). The hydroxamic acids were detected with acidic ferric chloride (Schweet *et al.* 1957). The R_F values for the hydroxamic acid cochromatographed with authentic material were: (*a*) 0·30, (*b*) 0·35 and (*c*) 0·60.

Substrate specificity. Table 4 demonstrates the absolute specificity of the purified enzyme preparation for amino acids with the D-configuration. Preliminary results (Baddiley & Neuhaus, 1959) with the relatively insensitive hydroxamate assay had indicated that this enzyme preparation was specific for D-alanine; however, specificity studies with the more sensitive exchange reaction demon-

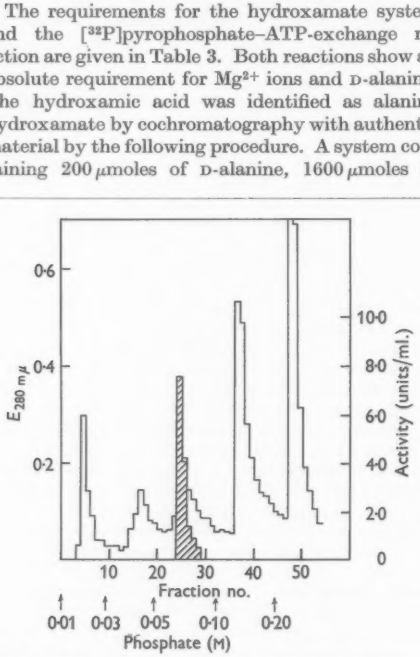

Fig. 3. Fractionation of D-alanine-activating enzyme on calcium phosphate. The shaded area represents the enzymic activity, expressed as units/ml., and the open area represents the protein concentration ($E_{280\,m\mu}$). The hydroxamate assay was performed on one-half scale.

Table 1. *Distribution of* D-*alanine-activating enzyme in various bacteria*

The hydroxamate assay was used without addition of inorganic pyrophosphatase, and the exchange assay was performed as described in the Experimental section. All extracts were dialysed for 18 hr. against 0·01 M-phosphate buffer, pH 6·8.

	Hydroxamate formation (μmoles/hr./mg. of protein)		[^{32}P]Pyrophosphate–ATP exchange (μmoles/hr./mg. of protein).
	D-Alanine	L-Alanine	D-Alanine
L. arabinosus	0·94	0·09	4·63
L. casei	3·90	0	13·63
B. subtilis	0·28	0·10	1·79
S. aureus H	0·53	0	4·72

Table 2. *Purification of* D-*alanine-activating enzyme*

Purification step	Volume (ml.)	Activity (units)	Specific activity (units/mg. of protein)	Yield (%)
1. Extraction	50	1200	1·37	100
2. Protamine sulphate treatment	54	1050	1·95	88
3. Ammonium sulphate fractionation	5·4	680	3·95	57
4. Column eluate	5·2	34·8	29·8	15*

* The results for step 4 represent a typical experiment on a 1 ml. sample from step 3 (125 units). The yield is calculated on the assumption that the whole ammonium sulphate fraction was chromatographed.

strated significant activity towards D-α-amino-*n*-butyric acid (14·6 %) and D-serine (7·5 %). In contrast with the absolute specificity for amino acids with the D-configuration, it appears that slight modifications introduced into the β-carbon atom of D-alanine are possible. Although it is not established that the D-alanine-activating enzyme is responsible for the activation of these two amino acids, it seems likely that they are activated by this enzyme by virtue of their similarity in structure.

Of the nucleotide triphosphates tested only ATP could catalyse the pyrophosphate–ATP exchange effectively; triphosphates of uridine, cytidine and guanosine showed less than 5 % of the activity of ATP. ADP had 10 % of the activity observed for ATP; a slight contamination of the ADP with ATP or the presence of myokinase could account for this activity. According to Pabst Laboratories (1959), ADP contains a trace of ATP.

pH *optima*. As illustrated in Fig. 4, the pH optimum for the hydroxamate system is 7·0–8·0, whereas the pH optimum for the [^{32}P]pyrophosphate–ATP-exchange reaction is 7·5–8·0.

Effect of pyrophosphatase and pyrophosphate. Purified preparations of this enzyme were markedly stimulated by adding yeast inorganic pyrophosphatase. As illustrated in Table 5, only 41 % of the maximum activity was observed in incubation for 1 hr. if the pyrophosphatase was omitted. Addition of 0·1 μmole of pyrophosphate in the absence of pyrophosphatase resulted in a reduction to 29 % of the maximum activity. In order to observe maximum activity with all preparations, including the crude extract, it was necessary to add pyrophosphatase.

Effect of D-alanine, ATP, pyrophosphate, hydroxylamine, and Mg^{2+} concentration on the hydroxamate system and on the exchange reaction. The concentration of D-alanine required to give one-half maximal activity in the hydroxamate system is approximately 0·4 M. Fig. 5 (curve *A*) shows the effect of D-alanine on the rate of the [^{32}P]pyrophosphate–ATP-exchange reaction. From a Line-

Table 3. *Requirements for the enzymic activation of* D-*alanine*

The hydroxamate assay with 200 μg. of enzyme preparation was used without added inorganic pyrophosphatase. The exchange assay was carried out with 50 μg. of enzyme preparation.

Modification to system	Hydroxamate formation (μmoles/hr./mg. of protein)	[^{32}P]Pyrophosphate–ATP exchange (%)	[^{32}P]Pyrophosphate–ATP exchange (μmoles/hr./mg. of protein)
Complete	3·5	15·7	28·0
Without Mg^{2+} ions	0	0·30	—
Without ATP	0	—	—
Without D-alanine	0	0·17	—
Without D-alanine; with L-alanine (100 μmoles)	0	0·26	—
Complete (boiled)	0	0·33	—

Table 4. *Specificity of* D-*alanine-activating enzyme*

The hydroxamate assay was carried out with 23 μg. of purified enzyme on one-half scale. In the exchange assay, series *A* contained 20 μg. of protein, whereas series *B* contained 23 μg. of protein.

Addition	Concn. of substrate (mole/l.)	Hydroxamate formation (μmoles/hr./mg. of protein)	[^{32}P]Pyrophosphate–ATP exchange (%) *A*	*B*
None	—	0	0·22	0·18
D-Alanine	0·100	25·0	20·80	35·40
L-Alanine	0·100	0	0·22	—
D-α-Amino-*n*-butyric acid	0·100	1·3	3·26	5·27
L-α-Amino-*n*-butyric acid	0·100	0	—	0·17
D-Serine	0·100	0	1·71	2·90
L-Serine	0·100	0	—	0·40
D-Phenylalanine	0·100	—	0·50	—
L-Phenylalanine	0·100	—	0·34	—
D-Glutamic acid	0·104	—	0·19	—
meso-α-ε-Diaminopimelic acid	0·012	—	0·19	—
DL-Alanyl-DL-alanine	0·100	0	0·28	—
β-Alanine	0·100	0	—	—
Glycine	0·094	0	—	—
Acetate (sodium)	0·100	0	—	—
DL-Valine	0·108	0	—	—

Table 5. *Effect of pyrophosphatase and pyrophosphate on the formation of* D-*alanine hydroxamate*

The complete system contained: 50 μmoles of tris–HCl buffer, pH 7·4; 5 μmoles of $MgCl_2$; 5 μmoles of neutralized ATP; 50 μmoles of D-alanine; 20 μg. of purified enzyme; 400 μmoles of hydroxylamine, pH 7·4; water to a total volume of 0·50 ml. The tubes were incubated for 60 min. at 37°. The reaction was stopped and the colour developed by the method of Cormier & Novelli (1958).

Modification to system	Activity (μmole/hr.)	Activity (% of max. rate)
Complete	0·590	[100]
Without pyrophosphatase	0·250	41
Without pyrophosphatase; with 0·1 μmole of pyrophosphate	0·175	29
Without pyrophosphatase; with 1 μmole of pyrophosphate	0·075	12

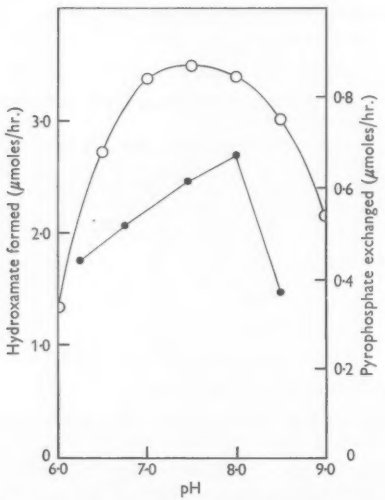

Fig. 4. Effect of pH on the rate of D-alanine activation. In the hydroxamate assay (○) the incubations contained: 10 μmoles of $MgCl_2$; 1·2 mg. of enzyme purified through step 3; 9·5 μmoles of neutralized ATP; 100 μmoles of D-alanine; 100 μmoles of tris–HCl buffer and 800 μmoles of hydroxylamine adjusted to the specified pH; water to a total volume of 1 ml. Controls incubated without D-alanine at the specified pH were used to correct these results. In the exchange assay (●) the following three components were adjusted to the specified pH: 25 μmoles of tris–malcate buffer, 9 μmoles of ATP and 5·3 μmoles of [^{32}P]-pyrophosphate. In addition, each tube contained: 18 μg. of purified enzyme, which had been preincubated with 1 μmole of glutathione for 15 min.; 100 μmoles of D-alanine; 10 μmoles of $MgCl_2$; water to a total volume of 1 ml. The tubes were incubated for 30 min. at 37°.

weaver & Burk (1934) plot of these results (Fig. 5, curve *B*) a K_m for D-alanine was determined (70 mM).

Much lower concentrations of ATP and pyrophosphate are required for maximal activity. Fig. 6 illustrates the effect of [^{32}P]pyrophosphate concentration on the exchange reaction. The K_m for pyrophosphate calculated from a Lineweaver–Burk plot is 0·13 mM. In the hydroxamate assay the maximum activity is obtained when the concentrations of ATP and Mg^{2+} ions are approximately equimolar. In Fig. 7 the effect of ATP concentration with equimolar Mg^{2+} ion concentration on the exchange reaction is shown. The concentration of ATP required for one-half maximal velocity is 2·5 mM. This agrees with the value

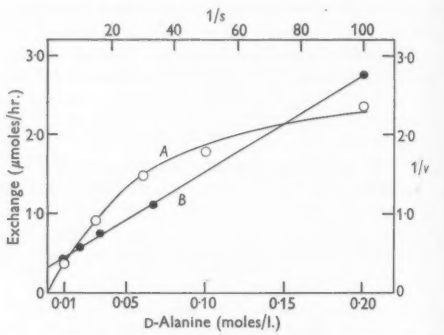

Fig. 5. Effect of D-alanine concentration on the [^{32}P]-pyrophosphate–ATP-exchange reaction (curve *A*) and the Lineweaver–Burk plot of these results (curve *B*); 20 μg. of protein was used.

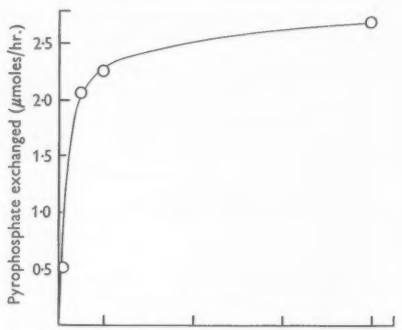

Fig. 6. Effect of [^{32}P]pyrophosphate concentration on the exchange reaction; 23 μg. of purified enzyme was used.

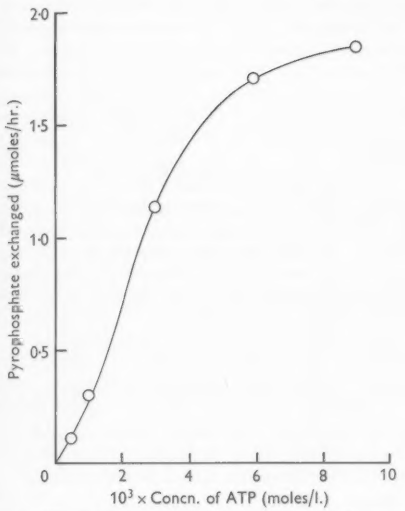

Fig. 7. Effect of ATP concentration on the [^{32}P]pyrophosphate–ATP-exchange reaction; 23 μg. of protein and 3·5 μmoles of [^{32}P]pyrophosphate were used. The concentration of $MgCl_2$ was equimolar with ATP.

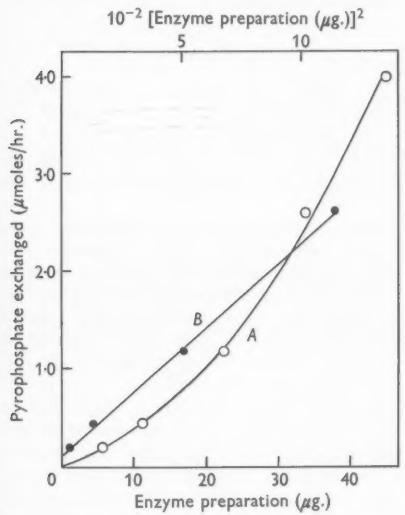

Fig. 8. Effect of enzyme concentration on the [^{32}P]pyrophosphate–ATP-exchange reaction in the absence of glutathione. Varying amounts of purified enzyme were used. In curve *A* the rate of pyrophosphate incorporation is plotted against the enzyme concentration, and in *B* against the square of the enzyme concentration.

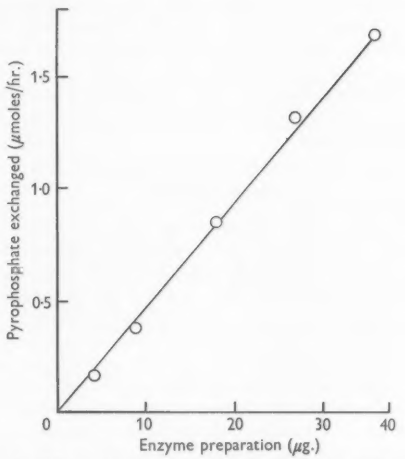

Fig. 9. Effect of enzyme concentration on the [^{32}P]pyrophosphate–ATP-exchange reaction in the presence of glutathione. The purified enzyme had been previously incubated with mM-glutathione for 15 min. at 37° (pH 6·8) before addition to the assay mixture.

obtained when the ATP concentration was varied in the hydroxamate assay. The maximum velocity in the hydroxamate system was observed when the hydroxylamine concentration was approximately 1 M: however, the hydroxamate assay was always carried out with 0·8 M-hydroxylamine.

Effect of glutathione on the enzyme. The effect of enzyme concentration on the [^{32}P]pyrophosphate–ATP-exchange reaction (Fig. 8, curve *A*), shows a non-linear relationship with the 25-fold-purified preparation. However, if the purified enzyme preparation is first incubated with mM-glutathione proportionality between activity and enzyme concentration is observed (Fig. 9). The amount of reactivation with glutathione appeared to depend on the age of the ammonium sulphate fraction (step 3) before chromatography on calcium phosphate. The maximum amount of activation observed in one preparation was fourfold; this was obtained by previous incubation for 15 min. with 10 mM-glutathione, pH 7·8. The effect was not observed with preparations that had been purified through step 3. From the data presented in Fig. 8 (curve *A*) the velocity of the exchange reaction is proportional to the square of the enzyme concentration.

Effect of possible inhibitors on the enzyme. The purified preparations contained approximately 2–3 % of nucleotide material, as determined by the method of Warburg & Christian (1941). Treatment of the purified preparation with ribonuclease

produced no significant inhibition in either the hydroxamate system or the exchange reaction. The possibility was considered that penicillin or chloramphenicol might affect this enzyme. However, mM-chloramphenicol and 10 mM-benzylpenicillin had no effect on the activation of D-alanine in the exchange assay.

DISCUSSION

The available evidence on the specificity of amino acid-activating enzymes indicates the occurrence of separate enzymes for each amino acid. The demonstration of an enzyme which activates D-alanine lends new support to this proposal.

Snell *et al.* (1955) concluded that the amount of D-alanine in intracellular protein of *L. casei* and *S. faecalis* is less than one-fifteenth of that present in the cell wall. Thus since the major fraction of the D-alanine is localized in the wall it seems likely that the D-alanine-activating enzyme is primarily concerned with the biosynthesis of cell-wall material rather than with the synthesis of intracellular protein.

Moreover, Snell *et al.* (1955) observed that the D-alanine required by *L. arabinosus* and *S. faecalis* that were grown in media deficient in vitamin B_6 could be replaced in part by D-α-amino-*n*-butyric acid. L-α-Amino-*n*-butyric acid and all other related amino acids were inactive. The D-alanine-activating-enzyme preparation had a slight action on both D-α-amino-*n*-butyric acid and D-serine. This suggests an intimate relationship between the growth experiments and the specificity of this enzyme. The response of the deficient microorganism to D-serine has not been examined.

The D-alanine-activating enzyme is similar in many respects to other amino acid-activating enzymes that have been characterized (Davie *et al.* 1956; van de Ven *et al.* 1958; Schweet & Allen, 1958); however, it differs in requiring a relatively high concentration of amino acid for maximal activity. No explanation is offered for this observation, but alanine racemase from *S. faecalis* also has a rather high K_m for alanine (8·5 mM; Wood & Gunsalus, 1951).

Novelli (1958) has observed a wide variation in the ability of bacterial extracts to catalyse amino acid-dependent [^{32}P]pyrophosphate–ATP-exchange reactions and the formation of hydroxamic acids. Webster (1959) proposed that this difference in activity towards specific amino acids might be the result of either a preferential inactivation of some of the amino acid-activating enzymes or a variation in their pH optima. The characteristics of the D-alanine-activating enzyme suggest another possible explanation for the low activities observed in some cases. If the K_m values of some of the enzymes are high then routine activation tests at a fixed amino acid concentration might be inadequate for demonstrating such enzymes.

In contrast with the high K_m for D-alanine, this enzyme exhibits a low K_m for pyrophosphate and ATP. The stimulation of the purified preparation by glutathione suggests the presence of an essential sulphydryl group on the enzyme. The purified enzyme preparations contain 2–3 % of a nucleotide material. With the L-alanine-activating system from rabbit liver, Ogata & Nohara (1957) found a reduction in activity after treatment with ribonuclease. In contrast with these results the purified tryptophan-activating enzyme (Davie *et al.* 1956) is not dependent on the 3–4 % of nucleotide material which it contains. In addition, the tyrosine-activating enzyme (Schweet & Allen, 1958) does not contain any detectable nucleotide. When the D-alanine-activating enzyme was treated with ribonuclease there was no significant decrease in its activity. This suggests that ribonucleic acid is not an essential factor in the activation of D-alanine.

The data which are presented in this paper are consistent with the formation during the activation of D-alanine of an enzyme–AMP–amino acid complex as shown in reaction 1. It is possible that this system may constitute the first step in the introduction of D-alanine into either teichoic acid or the muramic acid–peptide fraction of cell walls.

SUMMARY

1. An amino acid-activating enzyme for D-alanine has been detected in *Lactobacillus arabinosus*, *L. casei*, *Bacillus subtilis* and *Staphylococcus aureus* H. It has been purified 25-fold from ultrasonic extracts of acetone-dried cells of *L. arabinosus*.

2. The enzyme catalyses a [^{32}P]pyrophosphate–adenosine triphosphate (ATP) exchange in the presence of D-alanine and Mg^{2+} ions and the formation of D-alanine hydroxamate in the presence of ATP, Mg^{2+} ions, hydroxylamine and D-alanine. These reactions are consistent with the formation of an adenosine monophosphate–D-alanine anhydride in combination with the enzyme.

3. In the [^{32}P]pyrophosphate–ATP exchange the K_m for D-alanine is 70 mM, and for inorganic pyrophosphate it is 0·13 mM. The concentration of ATP required for one-half maximal velocity is 2·5 mM. The purified enzyme preparation is specific for D-amino acids. In addition to D-alanine this preparation catalyses a small but significant [^{32}P]-pyrophosphate–ATP exchange in the presence of D-α-amino-*n*-butyric acid (14·6 %) and D-serine (7·5 %).

4. The properties of this enzyme have been

compared with those of other amino acid-activating enzymes. In addition, its possible significance in the biosynthesis of bacterial cell-wall components is discussed.

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The Formation of Fibrils from Collagen Solutions*

1. THE EFFECT OF EXPERIMENTAL CONDITIONS: KINETIC AND ELECTRON-MICROSCOPE STUDIES

By G. C. WOOD† and M. K. KEECH‡
Department of Medicine, University of Leeds

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The idea has grown over the last decade that study of the precipitation of collagen fibrils from collagen solutions *in vitro* can provide some insight into the mechanism of fibrillogenesis *in vivo*. The formation and growth of collagen fibrils in tissue cultures has been widely studied by electron microscopy (Porter & Vanamee, 1949; Porter, 1951; Jackson, S. F., 1954*a*, *b*, 1955, 1956; Wassermann, 1954). Although it still seems to be an open question whether the fibrils are initially formed within or outside the fibroblasts, these studies indicate that the fibrils grow by accretion of soluble collagen present in the ground substance.

Soluble collagens may be extracted from young collagenous tissues by cold neutral-salt solutions, slightly alkaline buffers, dilute organic acids or organic acid buffers. Typical collagen fibrils may be precipitated, from solutions obtained, by suitably altering the experimental conditions (see Jackson, 1958*a*, for a review). They may also be formed by adding to the collagen solutions low concentrations of certain mucopolysaccharides and these observations have led to suggestions that mucopolysaccharides, abundant in developing connective tissue, might play an important role in the organization of soluble collagen precursors into fibrillar form (Meyer, 1946, 1955–56; Gross, 1956). There is, however, no strong evidence that their presence is essential for the formation of collagen fibres *in vivo* (Jackson, 1958*b*). Gross, Highberger & Schmitt (1955) and Jackson & Fessler (1955) have shown that collagen may be precipitated in the 640 Å striated form typical of native collagen, in the absence of added mucopolysaccharide, suggesting that these substances may not be important in this stage of the formation of collagen fibres. As indicated by Gross (1956), they may, however, play a part in the further organization of the fibrils.

Like the studies of fibril growth *in vivo*, most of the work on precipitation of collagen *in vitro* has been largely concerned with the appearance of the material in the electron microscope. Much of it has been qualitative and, except for the early work of Vanamee & Porter (1951), no systematic study has been made of the factors affecting precipitation, although Randall, Booth, Burge, Jackson & Kelly (1955) have enumerated the various factors to be considered.

Little attention has been paid to the kinetics of collagen precipitation. While the present work was in progress, however, Gross (1956), Gross & Kirk (1958) and Bensusan & Hoyt (1958) reported observations on factors affecting the rate of precipitation of collagen fibrils from neutral-salt solutions. The course of the precipitation was followed by measuring the increase in opacity of the system. In the present study a similar technique has been applied to a systematic study of the effect of experimental conditions (pH, ionic strength, temperature and collagen concentration) on the rate of fibril formation. Parallel to the rate studies the morphology of the collagen precipitates has been investigated by electron microscopy, the aims being to ascertain that the precipitates consisted of fibrils bearing 640 Å cross-striations typical of native collagen and to see how the size of the ultimate fibrils varied with the rate of precipitation under different conditions. The results provide a basis for formulating a kinetic mechanism which is treated in greater detail in the next paper (Wood, 1960*a*).

It seems likely that, as suggested by Gross (1958), solutions prepared by the different extractive procedures differ mainly in the state of aggregation of the dissolved collagen particles and that in each case the process of precipitation is essentially the same. In the present study acid-soluble collagen has been used, prepared in a form substantially free of mucopolysaccharide. The further problem of the effect of added mucopolysaccharides on fibril formation is considered by Wood (1960*b*).

A preliminary account of some of this work has been published (Wood, 1958).

* A paper by one of us (G.C.W.) based on work described in this and the following two papers gained second place in the Ciba Foundation's Awards, 1959, for Research Relevant to the Problems of Ageing.

† Nuffield Gerontological Research Unit.

‡ Empire Rheumatism Council Research Fellow.

EXPERIMENTAL

Materials

Purified calf dermis. Fresh calf skin was defatted mechanically and cut into strips. Hair, epidermis and remaining subcutaneous tissue were removed by dissection and the strips chopped into small pieces. These were passed through a power mincer, solid CO_2 being used as refrigerant, and homogenized with 3 vol. of water and freeze-dried. Most of the contaminating proteins, mucopolysaccharide and some soluble collagen were removed by neutral-salt extraction as follows: 50 g. of dermis was stirred gently with 650 ml. of 0·2M-NaCl, pH 7·0, for 16 hr. Undissolved material was separated by centrifuging and extracted again for 16 hr. with 500 ml. of 0·2M-NaCl. The residue was treated twice in the same way with 1·0M-NaCl, pH 7·0, and once with saturated NaCl. The final residue was washed with water until the washings were free of salt, suspended in water and freeze-dried. All the operations were carried out at 0–4° and the resulting purified dermis was stored at this temperature. For all the experiments described in this paper a single preparation of purified dermis (dermis X) was used.

Collagen solutions. Purified dermis (1 g.) was mixed with 60 ml. of 0·5M-acetic acid and allowed to stand for 16 hr. with occasional gentle stirring. The mixture was filtered through a coarse sintered-glass filter and the clear filtrate dialysed for a total period of 48 hr. against four changes of 2 l. of 0·1M-NaCl containing 0·005M-sodium acetate buffer, pH 4·2. The resulting viscous solution was then centrifuged for 45 min. at 20 000 *g*. All the operations were carried out at 0–4° and the collagen solution was stored at this temperature after its concentration had been adjusted to 0·1% by dilution with cold 0·1M-NaCl. Analytical data for a typical solution are given in Table 1. Assuming that the hydroxyproline content of collagen is 8·2 g. of amino acid N/100 g. of N (Bowes, Elliott & Moss, 1955), the hydroxyproline content of the solution indicates that about 93% of the dissolved protein is collagen.

Examination of a dried collagen solution prepared from another similar sample of purified dermis showed (Plate 1, photograph 1) that it consisted of non-striated filaments of varying size. No striated collagen fibrils were seen.

Analytical methods

Hydroxyproline was determined by the method of Neuman & Logan (1950) and nitrogen by the micro-Kjeldahl method. Hexosamine was estimated as glucosamine by the method of Boas (1953), hexose by the anthrone method of Scott & Melvin (1953) and phosphorus by the method of King (1932). Routine protein estimations were done by the method of Lowry, Rosebrough, Farr & Randall (1951). Viscosities were measured at 25° in Ostwald-Fenske viscometers with flow times for water of about 6 min.

Table 1. *Analytical data for soluble collagen prepared from purified calf dermis*

Component	g./100 g. of total N
Hydroxyproline N	7·6
Hexosamine (as glucosamine)	1·7
Hexose (as glucose)	1·3
Phosphorus	0·13

Methods of following the course of precipitation

Turbidity method. In a typical experiment 1·5 ml. of collagen solution was allowed to stand in a spectrophotometer cell (1 cm. light path) at 25° for 1·5 hr. At zero time precipitation was initiated by adding 1·7 ml. of buffer solution (pH range 6–8) previously equilibrated at 25° and mixing the cell contents thoroughly with a polythene stirrer similar to that described by Dixon (1954). The stirrer was withdrawn and E at 400 mμ measured at intervals throughout formation of the gel-like precipitate. A Unicam SP. 500 spectrophotometer fitted with a constant-temperature cell holder was used. It was modified by placing a pin hole (approx. 0·5 mm. diameter) in front of the dark current shutter in order to minimize the amount of forward-scattered light reaching the photocell. One result of this was to increase the apparent extinction of a gel by 15–20%.

$NaOH$–KH_2PO_4 buffers were used, the total phosphate content being maintained constant so that its value in the reaction mixture was 0·04M. When the effect of pH was studied the ionic strength was maintained by adjusting the NaCl content of the buffer. To study the effect of ionic strength (I) the NaCl content of the added buffer was varied.

In all experiments the collagen solution was equilibrated at a concentration of 0·1% at 25°, well below the thermal-transition temperature reported by Doty & Nishihara (1958). For experiments at temperatures other than 25° the buffer was equilibrated at the working temperature, added to the collagen solution at 25° and the mixture brought as quickly as possible to working temperature. For experiments at different collagen concentrations different volumes of 0·1% collagen solution were equilibrated and the volume and composition of the added buffer adjusted so that the final volume and salt content were the same.

Fig. 1 shows progress curves for two successive experiments which give an indication of the reproducibility. Other work showed that the curves changed gradually with the age of the collagen solution. For this reason only solutions less than 10 days old were used and progress curves are strictly comparable only when the experiments were done on the same day. Some variation from one preparation of collagen solution to another was observed.

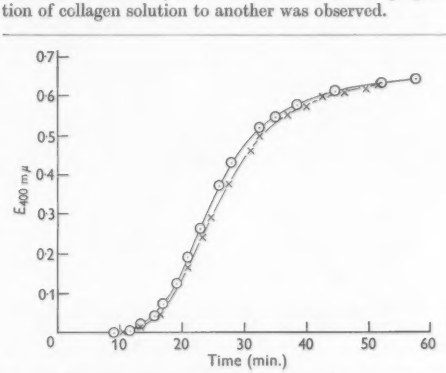


Fig. 1. Progress curves showing the course of precipitation in two successive, identical, experiments. Initial collagen concn., 0·05%; temp., 25°; I 0·23; pH 7·1.

Calibration of the turbidity method by a chemical procedure. Since the relationship between extinction and amount of precipitate cannot be predicted theoretically it was necessary to calibrate the method by actually measuring the amount of precipitate. Simultaneously with a turbidity experiment several precipitations were allowed to proceed, under the same conditions, in 10 ml. centrifuge tubes. After different known reaction times 0·2 ml. of 0·5 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) hydrochloride in 0·2 M-NaOH–KH_2PO_4 buffer, pH 7, was added. The collagen gel was compressed to the bottom of the tube, centrifuged and the protein content of the supernatant estimated, enabling the extent of precipitation to be calculated and compared with the extinction of the gel at the corresponding time. Addition of tris to the reaction mixture prevented any significant further precipitation during the time required for the estimation and dissolved less than 3% of the collagen already precipitated.

Fig. 2 shows the relationship between the extinction of a collagen gel at different times during its formation and the corresponding extent of precipitation, p, given by $p = (C_0 - C)/(C_0 - C_s)$, where C is the collagen concentration. C_0 and C_s are the initial and final collagen concentrations respectively. Thus $p = E/E_\infty$ to a close approximation, where E is extinction and E_∞ the extinction at infinite time. Similar straight lines were obtained in several experiments under different conditions. Extinction is therefore a reliable measure of the extent of precipitation and, in view of its convenience and precision, the turbidity method has been used for the results described below.

Electron-microscope methods

Preparation and examination of collagen precipitates. The collagen solutions and buffers were the same as those used for the rate studies and as far as possible the procedure for preparing the precipitates was also the same.

Glass slides were equilibrated in Petri dishes maintained at working temperature (25°, 37° or bench temperature—approx. 21°). After the collagen and buffer solutions had been equilibrated at the required temperatures and mixed as described in the rate studies, approximately 0·5 ml. of the mixture was immediately transferred to duplicate slides, spread to make pools 1–2 mm. deep and immediately replaced in the Petri dishes. Extra buffer in tinfoil cups was put in each dish to minimize evaporation of water from the reaction mixtures.

After 24 hr. the slides were dried in 10–15 min. in a stream of air at room temperature. They were gently washed with water to remove buffer salts, dried again, shadowed with gold–palladium at an angle of 5° and covered with a thin film of collodion. After scoring the film into squares, dermal components were removed by trypsin digestion overnight at room temperature. The loosened squares were then peeled from the slide, washed in a dish of water and picked up on electron-microscope grids. The final preparations thus consisted of metal-shadowed replicas of the surfaces of the undisturbed, washed, dried precipitates. These were examined in a Siemens electron microscope, type UM 60C, large areas of several grids from each preparation being scrutinized.

No antibacterial agents were used but, by employing only freshly-made collagen and buffer solutions, bacterial contamination was avoided.

Measurement of fibril width. In conformity with present-day terminology the term 'fibril' denotes a fibrous structure which is apparently undivided at the magnification used.

Fibril width was measured on the electron-microscope plates with a scaled Leitz eyepiece (magnification ×8¹. Only single fibrils were measured, aggregates of fibrils being disregarded. It is assumed that the former provide a representative sample of all the fibrils.

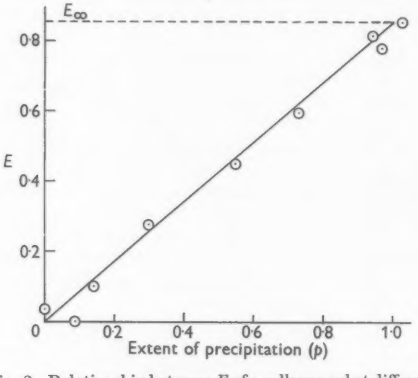


Fig. 2. Relationship between E of a collagen gel at different times during its formation and the extent of precipitation (p) determined chemically. E_∞, final extinction. Initial collagen concn., 0·05%; temp., 25°; I 0·23; pH 7·1.

RESULTS

Effect of thermal and mechanical agitation of the collagen solutions

Fig. 3 shows how precipitation curves for one collagen solution varied with the time of equilibration of the solution at 25° and indicates that 1·5 hr. is required to attain true equilibrium. This is much greater than the time required to attain temperature equilibrium and must be due to some change in the state of the dissolved collagen. This change is accompanied by a change of viscosity, as indicated in Fig. 3. Similar effects were caused by stirring the extract at 0°, when they were most pronounced in dilute solutions and were partly reversed by allowing the solution to stand for 16 hr. at 0°. These phenomena serve to emphasize the necessity of adhering to the strict routine already described in performing the precipitation experiments, minimizing mechanical agitation of the collagen and always using an equilibration time of 1·5 hr.

Effect of varying collagen concentration, pH, ionic strength and temperature

Rate of precipitation. In Figs. 4 and 5 the four sets of precipitation curves show the effect of each of the variables in turn.

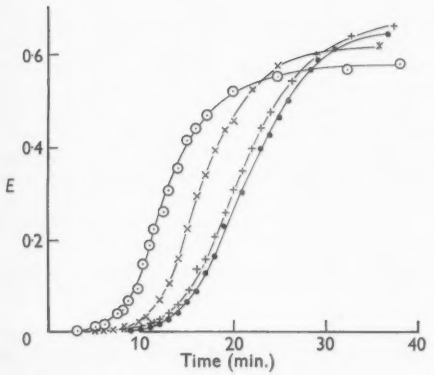


Fig. 3. Effect of varying time of equilibration (t_e) on subsequent precipitation. t_e: ⊙, 0·5 hr.; ×, 1·0 hr.; ●, 1·5 hr.; +, 3·25 hr. Relative viscosities ($\eta_{rel.}$) of corresponding equilibrated collagen solutions: 2·98, 2·84, 2·75 and 2·73 respectively. During precipitation: initial collagen concn., 0·05%; temp., 25°; I 0·23; pH 7·1.

All the precipitation curves have the same shape, consisting of a lag period, during which no precipitation was observed, followed by a sigmoid portion or growth period. Although it cannot be determined precisely, the extent of the lag period is approximately given by $t_{0\cdot005}$, the time taken for p to rise to the value 0·005. A measure of the rate of growth is given by the half-growth time ($t_{0\cdot5}$), i.e. time taken for p to rise from 0·005 to 0·5. These two quantities are given in Tables 2–4 for the above data, together with the results of some experiments not given in graphical form. E_∞/C_0 is also shown in the tables. Since $C_e \simeq 0$ in all experiments (i.e. the collagen was completely precipitated) the results are comparable within each set.

In all cases a change of experimental conditions which shortened the lag period also accelerated subsequent precipitation and vice versa. Precipitation was accelerated by increasing the collagen concentration and by increasing the temperature. It was retarded by increasing the ionic strength at pH 7 and 8; at pH 6, change of ionic strength from 0·13 to 0·23 retarded precipitation but further increase to 0·31 accelerated it. When pH was varied at ionic strength 0·23 the rate of precipitation showed a minimum at pH 7·0–7·5.

Acceleration of precipitation by changing the ionic strength or temperature was accompanied by

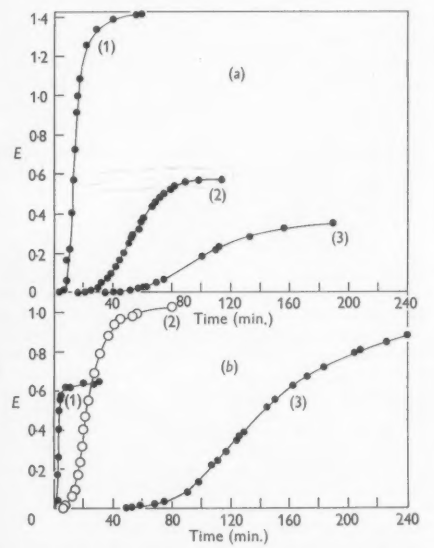


Fig. 4. Effect of varying experimental conditions on precipitation. (*a*) Different initial collagen concentrations (C_0): (1) 0·084; (2) 0·028; (3) 0·017%. (*b*) Different temperatures: (1) 37·0°; (2) 25·0°; (3) 20·2°. Except for the single variables in each set, C_0 was 0·05%; temp., 25°; I 0·23; pH 7·1. The two sets of experiments were done on different collagen solutions.

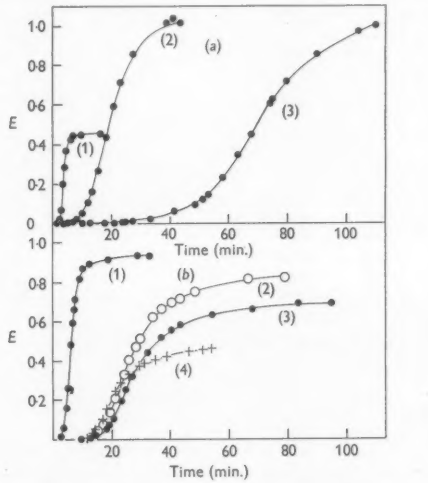


Fig. 5. Effect of varying experimental conditions on precipitation. (*a*) Different ionic strengths: (1) I 0·13; (2) I 0·23; (3) I 0·31. (*b*) Different pH values: (1) pH 6·0; (2) pH 7·1; (3) pH 7·5; (4) pH 8·0. Except for the single variables in each set, C_0 was 0·05%; temp., 25°; pH 7·1; I 0·23. The two sets of experiments were done on different collagen solutions.

Table 2. *Rate of precipitation of collagen at different initial concentrations (C_0) of collagen*

Each set of data was obtained with a different preparation of collagen solution. Experimental conditions: pH 7·1; I 0·23; 25°. For definition of $t_{0·005}$, $t_{0·5}$ and E_∞ see text.

C_0 (%)	Lag period, $t_{0·005}$ (min.)	Half-growth time, $t_{0·5}$ (min.)	E_∞/C_0
0·084*	3	12	16·8
0·061	8	14	21·5
0·050	8	12	19·0
0·028*	20	35	15·8
0·017*	30	73	21·5
0·050	5	12	21·0
0·034	13	39	21·7
0·017	30	87	21·2

* Data correspond to curves in Fig. 4*a*.

Table 3. *Rate of precipitation of collagen at different values of* pH *and ionic strength*

Each set of data was obtained with a different preparation of collagen solution. C_0 0·05%; 25°. For definitions of $t_{0·005}$, $t_{0·5}$ and E_∞ see text.

pH	I	Lag period, $t_{0·005}$ (min.)	Half-growth time, $t_{0·5}$ (min.)	E_∞/C_0
6·0*	0·23	2	5	18·8
7·1*	0·23	8	20	16·6
7·5*	0·23	12	18	14·0
8·0*	0·23	9	13	9·2
6·0	0·23	2·5	2·7	18·0
7·1	0·23	6	10	18·0
8·0	0·23	5	8	9·2
7·0†	0·13	2	2	9·2
7·0†	0·23	5	15	21·6
7·0†	0·31	24	48	23·0
7·0	0·13	2	1·5	8·0
7·0	0·23	6	10	18·0
7·0	0·31	13	22	18·8
6·0	0·13	1	2·5	11·8
6·0	0·23	2	4·5	18·8
6·0	0·31	1	3	19·8
8·0	0·13	3	5	6·8
8·0	0·23	6	7	9·4
8·0	0·31	20	50	16·4

* Data correspond to curves in Fig. 5*b*.
† Data correspond to curves in Fig. 5*a*.

Table 4. *Rate of precipitation of collagen at different temperatures*

Experimental conditions: pH 7·0; I 0·23. For definitions of $t_{0·005}$, $t_{0·5}$, E_∞ and C_0 see text. Data correspond to curves in Fig. 4*b*.

Temp.	Lag period, $t_{0·005}$ (min.)	Half-growth period, $t_{0·5}$ (min.)	E_∞/C_0
20°	45	93	18·0
25	9	19	21·0
30	3	9	23·6
37	1	2	13·0

a decrease of E_∞/C_0. On the other hand, E_∞/C_0 fell continuously as pH was increased from 6 to 8 and does not seem to be correlated with the rate of precipitation in a simple manner. E_∞/C_0 was essentially independent of collagen concentration. Since the collagen was always completely precipitated, the difference of E_∞/C_0 observed in each set of experiments must be due to differences in the structure of the precipitates. The nature of these differences will become clear when the electron-microscope results are described.

Tables 2–4 also show the variation observed in precipitation behaviour from one preparation of collagen solution to another. In spite of this, the manner in which rate of precipitation varied with conditions was reproducible.

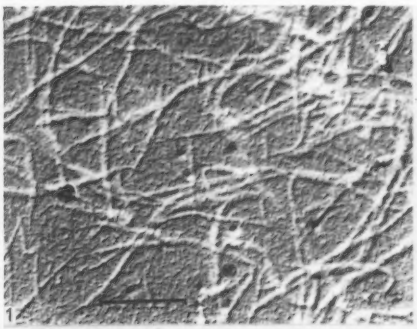
Electron-microscope results. Examination of the replicas under low power (× 300) showed that the precipitates consisted of fibrous networks whose coarseness varied with experimental conditions. These networks were seen under high power (× 10 000) to consist almost entirely of long fibrils bearing 640 Å striations (Plates 1 and 2, photographs 2–9) together with variable quantities of background filaments. The width of the fibrils varied with experimental conditions.

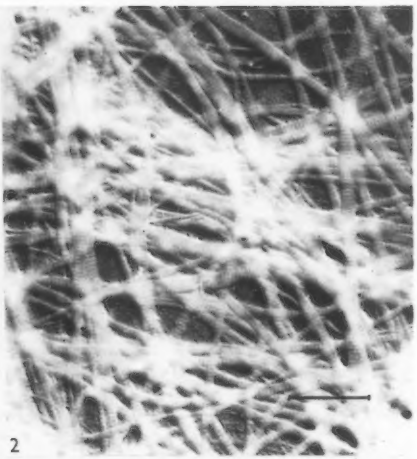
The fibrils showed varying degrees of lateral aggregation. Bundles of fibrils (e.g. Plate 1, photograph 3) occurred in most of the precipitates. Their component fibrils, usually 1000–1400 Å wide, were separate and the bundles, which frequently looked cylindrical, sometimes appeared to be twisted. Fibrils were sometimes observed side by side in one plane with their cross-striations in register. When wide fibrils were aggregated in this way, with

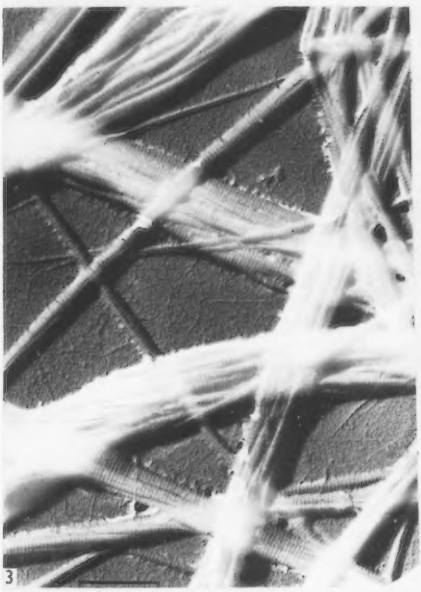
EXPLANATION OF PLATE 1

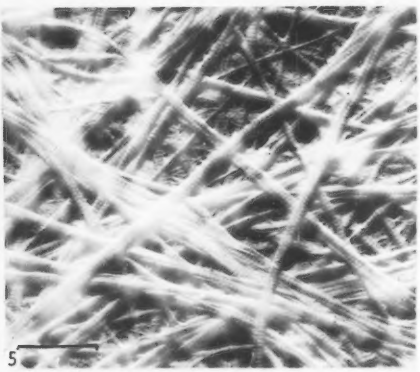
Plates 1 and 2. The electron micrographs are (except no. 1) of replicas of precipitates shadowed with gold–palladium. The scale on each photograph represents 1 μ. The striated structures exhibit 640 Å periodicity unless otherwise stated. Curves showing the rate of formation of the precipitates illustrated in photographs 2–4 and 7 and 8 are given in Figs. 4*c* and 9 respectively.

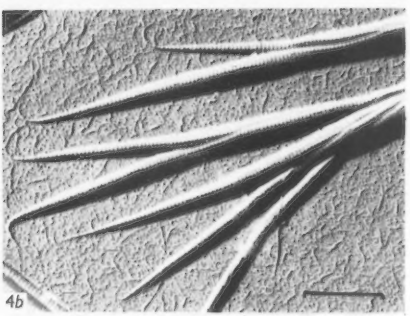
(1) The fibrous felt-work observed when a drop of the collagen solution was air-dried on the grid, washed and shadowed with chromium. (2–4) Precipitates prepared at different ionic strengths at pH 7·1, 25°. For distribution of fibril width see Fig. 6. (2) Dense deposit of fibrils with very little bundle formation, obtained at I 0·13. (3) Marked lateral aggregation of fibrils to form bundles at I 0·23. Few background filaments were seen. (4) Fibril bundles and acute fibril bends (4*a*) and tapered fibril ends (4*b*) observed at I 0·31. Some background filaments were seen. The bright borders to some of the fibrils are artifacts. (5) Precipitate prepared at I 0·23, 25°. For distribution of fibril width see Fig. 7. Dense deposit of fibrils with very little bundle formation, observed at pH 8·0.











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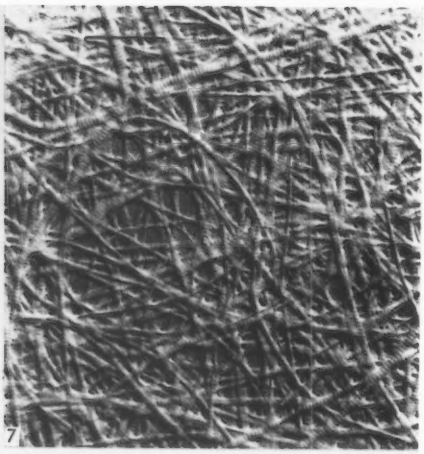
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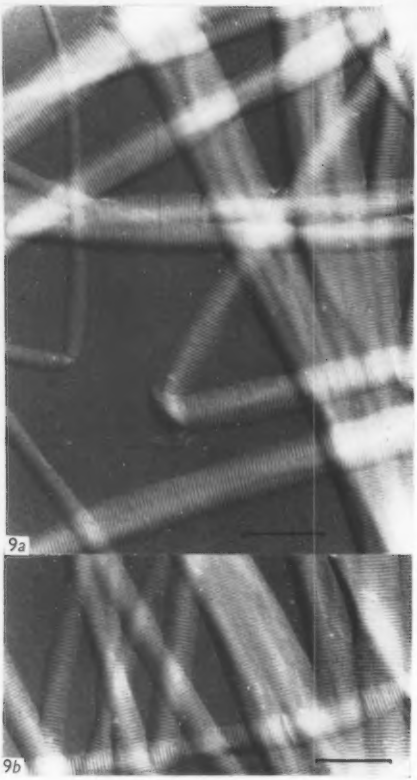
9a
9b

partial loss of identity, they gave the appearance of sheets (Plate 2, photograph 6) or compound fibres (Plate 2, photographs 9*a*, *b*). The above classification of lateral aggregation is arbitrary and there was in fact no sharp distinction between the various forms.

Table 5 summarizes the results of four sets of experiments designed to examine the effect of varying, in turn, each of the variables: ionic strength (*I*), pH and temperature. For each set a different preparation of collagen solution was used. The fibril-width data are taken from the histograms in Figs. 6–8 and Plates 1 and 2, photographs 2–9, show a representative set of electron-microscope photographs.

When ionic strength was varied at pH 7·1, 25°, the precipitate at *I* 0·13 appeared as a dense mat of discrete fibrils mainly 600–1200 Å wide (Plate 1, photograph 2). As *I* was increased to 0·23 and 0·31 the most frequent fibril width and the range of fibril width both increased (Fig. 6). Bundles were observed at *I* 0·23 (Plate 1, photograph 3) and at *I* 0·31, and at the latter ionic strength tapered fibril ends and acute fibril bends were seen (Plate 1, photographs 4*a*, *b*).

When pH was varied at constant *I* (0·23), 25°,

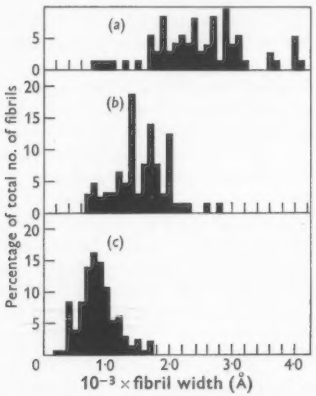

Fig. 6. Distribution of fibril width in precipitates prepared at different ionic strengths: (*a*) *I* 0·31; (*b*) *I* 0·23; (*c*) *I* 0·13. C_0, 0·05%; temp., 25°; pH 7·1. The number of fibrils measured was 72, 64 and 129 respectively. The data were obtained from the series of electron micrographs of which Plate 1 (2–4) gives examples.

EXPLANATION OF PLATE 2

(6) Precipitate prepared at *I* 0·23, 25°. For distribution of fibril width see Fig. 7. Sheet formation, which, together with compound fibres and bundles, was observed at pH 7·1. (7–9) Precipitates prepared at different temperatures at pH 7·1, *I* 0·23. For distributions of fibril width see Fig. 8. (7) Dense deposit of narrow fibrils obtained at 37°. No bundles were seen. Some of the narrower fibrils exhibited 220 Å striations. (8) Precipitate containing fibrils both singly and in bundles obtained at 25°. (9) Precipitate prepared at 21° showing wide fibrils, compound fibres and tendency to form sheets (9*b*). Acute fibril bends were also seen (9*a*).

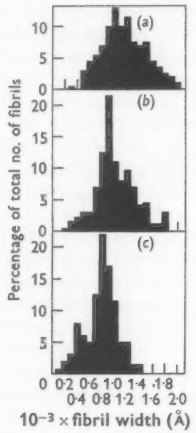

Fig. 7. Distribution of fibril width in precipitates prepared at different pH values: (*a*) pH 6·0; (*b*) pH 7·1; (*c*) pH 8·0. C_0, 0·05%; temp., 25°; *I* 0·23. The number of fibrils measured was 284, 190 and 146 respectively. The data were obtained from the series of electron micrographs of which Plates 1 and 2 (5–8) gives examples.

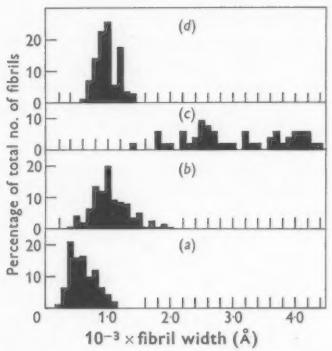

Fig. 8. Distribution of fibril width at different temperatures: (*a*) 37°; (*b*) 25°; (*c*) 21°; (*d*) 25° for 10 min. and then 37°. C_0, 0·05%; temp., 25°; *I* 0·23; pH 7·1. The number of fibrils measured was 140, 195, 51 and 56 respectively. The data were obtained from the series of electron micrographs of which Plate 2 (7–9) gives examples. Expts. (*a*), (*b*) and (*d*) were done on one collagen solution and (*c*) on another.

a dense mat of discrete fibrils mainly of 600–1200 Å width was observed at pH 8 (Plate, 1, photograph 5). As the pH was decreased to pH 7 and 6 the most frequent fibril width increased slightly (Fig. 7). Bundles were observed at pH 6 and 7, and at pH 7 compound fibres, sheets and acute fibril bends were observed (Plate 2, photograph 6). An additional feature at pH 8 was a network of fine filaments which in some areas partially obscured the fibrils.

In the third set of experiments (variation of temperature at pH 7·1, I 0·23) a dense mat of discrete fibrils was observed at 37° (Plate 2, photograph 7). The majority of the fibrils had 640 Å striations but the thinner one showed 220 Å periodicity. As the temperature was reduced the most frequent fibril width and the range of fibril width increased (Fig. 8). Bundles were observed in the 25° precipitate (Plate 2, photograph 8), whereas in the 21° precipitate compound fibres (Plate 2, photograph 9*b*), sheets and acute fibril bends (Plate 2, photograph 9*a*) were seen. Since the 21° experiment was done on a different collagen solution from that used for the 25° and 37° experiments the results are not strictly comparable, but the large difference in fibril width between the 25° and 21° precipitates is probably significant.

Thus in the range of conditions used decrease of temperature and pH and increase of ionic strength tend to increase the most frequent fibril width, increase the range of fibril width and displace it to higher values and increase the extent of lateral aggregation. The results of three experiments (Table 5) done under identical conditions on different preparations of collagen solution indicate the extent to which the morphology of the precipitates is reproducible.

Included in Table 5 are values of E_∞, the final extinction of collagen gels prepared simultaneously with the electron-microscope experiments from the same collagen solutions under identical experimental conditions. It has already been noted that variation of E_∞ with experimental conditions must be due to variations of the morphology of the precipitate, and Table 5 shows that E_∞ increases as the fibril width and extent of lateral aggregation increase. For certain experiments (Table 5) rate of precipitation curves were determined which correspond to the electron-microscope results and are those shown in Figs. 5*a* and 9.

Effect of temperature on the two stages of precipitation

The shape of the precipitation curves suggests that precipitation can be divided into two stages, the first represented by the lag period and the second by the sigmoid portion of the curve. It was of interest therefore to see how each of these steps responded to change of experimental conditions. In order to avoid the necessity for stirring the reaction mixture, temperature was chosen as the experimental variable.

Two identical samples of collagen solution were allowed to precipitate in the usual way, one at 25° (Fig. 9, curve 1), the other at 37° (Fig. 9, curve 2). As in other experiments, increasing the temperature accelerated precipitation and lowered E_∞. The corresponding electron micrographs are given in Plate 2, photographs 8 and 7, and the fibril-width distributions in Fig. 8. These have already been described and we need only reiterate the correlation between E_∞ and fibril width. A third reaction mixture was kept at 25° until the end of the lag period and its temperature then raised as quickly as possible to 37° (Fig. 9, curve 3). Thus although

Table 5. *Summary of electron-microscope data*

Each set of data was obtained with a different preparation of collagen solution. The fibril-width measurements are taken from Figs. 6–8.

pH	I	Temp.	Appearance under low power	Most frequent fibril width (Å)	Range of fibril width (Å)	Lateral aggregation (increasing extent) → Bundles	Compound fibres	Sheets	E_∞
7·1	0·13	25°	Fine	900	200–1700	±	–	–	0·46*
7·1	0·23	25	Coarse	1600	700–2800	+ + +	–	–	1·08*†
7·1	0·31	25	Coarse	2500	800–4100	+ +	–	–	1·15*
6·0	0·23	25	Coarse	1100	500–2000	+ +	–	–	0·74
7·1	0·23	25	Coarse	900	200–1800	+	+	+	0·69†
8·0	0·23	25	Fine	800	100–1400	±	–	–	0·63
7·1	0·23	25	Coarse	1000	400–2000	+ + +	–	–	0·90*†
7·1	0·23	37	Fine	500	200–1200	–	–	–	0·62*
7·1	0·23	21	Coarse	2500	1800–4800	–	+ +	+	—

* Precipitation curves determined on the same collagen solutions are shown in Figs. 5*a* and 9.

† These results were obtained under identical experimental conditions with three different preparations of collagen solution.

raising the temperature to 37° after the lag period accelerated subsequent precipitation, the value of E_∞ was identical with that observed with the temperature at 25° for the whole time. The corresponding electron micrographs and the fibril-width distribution (Fig. 8) were almost indistinguishable from the data obtained with the temperature at 25° throughout precipitation. The results of these experiments are summarized in Table 6. It appears therefore that under these conditions, at least, both sections of the overall precipitation are accelerated by increasing the temperature but that fibril width and hence E_∞ are determined during the lag period.

DISCUSSION

The electron-microscope approach, notably in the hands of Schmitt and co-workers (Schmitt, Gross & Highberger, 1955*a*, *b*; Gross, 1956; Schmitt, 1959), has led to the concept of collagen precipitation *in vitro* as the aggregation of rod-like collagen particles, whose presence in collagen solutions has been demonstrated (Boedtker & Doty, 1956; Doty & Nishihara, 1958; Hall, 1956; Hall & Doty, 1958). In spite of the wide variation of rate of formation, ultimate fibril width and extent of aggregation, all the precipitates observed in the present study consisted, almost entirely, of fibrils bearing striations with 640 Å periodicity. In all cases therefore the soluble-collagen particles must have aggregated in essentially the same manner; probably in the same manner as in the formation of collagen fibrils *in vivo*.

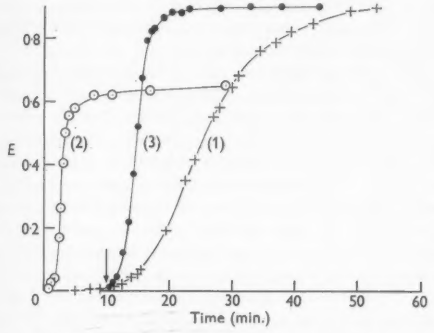

Fig. 9. Effect of temperature on the two steps of precipitation: (1) 25° throughout precipitation; (2) 37° throughout precipitation; (3) 25° during lag period, 37° after lag period. The arrow marks the end of the lag period. C_0, 0·05%; temp., 25°; I 0·23; pH 7·1.

Boedtker & Doty (1956) presented evidence, however, for the presence of aggregated material in some of their solutions, and Mathews, Kulonen & Dorfman (1954) suggested that reversible systems of aggregates occur in collagen solutions. Some of the collagen particles present in our solutions may not therefore have been discrete collagen molecules but molecular aggregates. The presence of aggregates in the cold collagen solutions used in the present work and their slow breakdown when the solutions were brought to 25° or were agitated mechanically might account for the change in viscosity and accompanying change of rate of precipitation (Fig. 3). Differences of rate of precipitation between different preparations of collagen solution and between samples of the same collagen solution of different ages might also be due to differences of extent of molecular aggregation.

Different methods of preparing collagen solutions may well result in starting material, for precipitation experiments, of different degrees of molecular aggregation, and it would not be surprising therefore if precipitation curves determined by Gross & Kirk's or Bensusan & Hoyt's procedures, which involve prior dialysis of the collagen against neutral buffers, differed in detail from

Table 6. *The two steps of precipitation*

The three experiments were done on the same collagen solution. Experimental conditions: pH 7·1; I 0·23; C_0, 0·05%; 25°. The distributions of fibril width are shown in Figs. 8*a*, *b*, *d*. They were obtained from the series of electron micrographs of which Plate 2, photographs 7 and 8 are examples. Values of E_∞, lag period ($t_{0\cdot005}$) and half-growth time ($t_{0\cdot5}$) are taken from the precipitation curves in Fig. 9. For definitions of E_∞, $t_{0\cdot005}$ and $t_{0\cdot5}$ see text.

Temp.	Appearance under low power	Most frequent fibril width (Å)	Range of fibril width (Å)	Lateral aggregation: Bundles	Lateral aggregation: Compound fibres	Lateral aggregation: Sheets	E_∞	$t_{0\cdot005}$	$t_{0\cdot5}$
25° throughout both steps	Coarse	1000	400–2000	+ + +	–	–	0·90	9	16
37 throughout both steps	Fine	500	200–1200	–	–	–	0·62	1	2
25 for first step (10 min.), 37 for second step (remainder)	Coarse	1000	600–1400	+ + +	–	–	0·90	—	6

curves determined in the manner described in this paper. The nature of the molecular aggregates present in solution is unknown but the occurrence of non-striated background filaments in some of the electron micrographs suggests that they may be filamentous in nature (Reed, 1957).

Further information on the mechanism of fibril formation results from correlation of the kinetic and electron-microscope approaches to the problem. In order that the results of the morphological studies should be comparable with those of the rate studies it was necessary to prepare the samples for examination in the electron microscope with the minimum of mechanical disturbance, for, as M. K. Keech (in preparation) has shown, the collagen gels are extremely sensitive to even mild mechanical stress. The electron micrographs indicate that, by replicating the undisturbed precipitate, the integrity of the individual fibrils was, as far as we can tell, maintained. In comparable experiments the final extinction of the collagen gel (E_∞) is correlated with the width of the fibrils, as seen in the electron microscope, and it is reasonable to assume that in other rate experiments for which we do not have comparable electron-microscope data, change of E_∞ is an indication of change of fibril width. Although precautions were taken to make the kinetic and electron-microscope results comparable the latter technique incurs the additional drying of the gel. It is possible that lateral aggregation of the fibrils is a result of the quite large forces which are well known to develop during such drying (see, for example, Anderson, 1956). We cannot say therefore whether or not lateral aggregates of fibrils are present in the gel state and in the following discussion attention is focused on fibril width. However, whether aggregation arises during precipitation or on drying, it was more evident in some precipitates than in others, being most pronounced when the most frequent fibril width was greater than 1000 Å.

When precipitation was accelerated by increasing the temperature, by lowering the ionic strength or by increasing pH from 7 to 8 (Figs. 4 and 5; Tables 3 and 4), the ultimate fibril width was decreased (Table 5). On the other hand when precipitation was accelerated by decreasing the pH from 7 to 6 (Fig. 5 and Table 3) the ultimate fibril width was increased (Table 5). When precipitation was accelerated by increasing C_0 (Fig. 4 and Table 2) the value of E_∞/C_0 remained approximately constant, which suggests that fibril width does not vary much with collagen concentration. Thus there is no simple relationship between rate of precipitation and fibril width. The reason for this becomes clearer when fibril formation is considered as aggregation of elongated particles (discrete rod-like molecules or long aggregates), for it would appear that there are two principal factors determining fibril width. First, fibril width will be partly determined by the number of fibrils: the larger the number of fibrils formed from a given amount of soluble collagen the smaller will be each fibril, and this might reasonably be expected to be correlated with the rate of fibril formation. The second factor arises from the way in which the particles aggregate. According to the ideas put forward by Schmitt and co-workers (Schmitt *et al.* 1955*a*, *b*; Gross, 1956; Schmitt, 1959), they aggregate with their long axes parallel to the axis of the ultimate fibril. They may do this with varying degree of overlap or relative proportion of side-by-side and end-to-end aggregation, which may depend on experimental conditions. The greater the degree of overlap or the greater the proportion of side-by-side aggregation the wider (and shorter) will the resulting fibril tend to be. The observed fibril width will be determined by both these factors, which need not necessarily act in the same direction, although both must be determined by the same interparticle forces.

The way in which rate of precipitation and E_∞ vary with temperature, collagen concentration and ionic strength (Figs. 4 and 5; Tables 2–4) is qualitatively similar to that observed by Bensusan & Hoyt (1958) and Gross & Kirk (1958). The retarding effect of increasing salt concentration at pH 7 and 8 suggests that the rate or manner (or both) of aggregation of the soluble collagen particles is controlled to a significant degree by electrostatic interaction between them. At pH 6, however, there appears to be a salting-out effect at high ionic strength in so far as precipitation at I 0·31 is faster than at I 0·23 (Table 3). A further indication of the importance of electrostatic interaction is provided by the variation of rate of precipitation with pH (Fig. 5 and Table 3). The acceleration resulting from decreasing the pH from 7·5 to 6·0 is consistent with a reduction in the net charge on the collagen as the isoelectric point is approached. The increase of rate on further removal of pH from the isoelectric point (from pH 7·5 to 8·0) is not, however, consistent with this simple electrostatic mechanism. Gross & Kirk (1958), also using phosphate buffers, observed the same acceleration at high pH when precipitation was slowed down to a measurable rate by adding low concentrations of urea. It is possible that change, with pH, of the binding of phosphate ions to the collagen is responsible for the increase of rate on going from pH 7·5 to 8·0. No optimum pH, such as that observed by Bensusan & Hoyt (1958), was observed in the range of pH covered. The ionic strength (0·23) was, however, higher than that (0·105) used by Bensusan & Hoyt, and it is known that the isoelectric point of collagen is strongly dependent on ionic strength

(Jackson & Neuberger, 1957). Moreover, Bensusan & Hoyt (1958) used a different buffer system and, as they point out, different anions can exert different effects on the rate of precipitation. The variation of fibril width with pH is similar to that observed by Vanamee & Porter (1951), although it should be noted that they used a different buffer system.

The shape of the precipitation curves suggests, as pointed out by Bensusan & Hoyt (1958), that precipitation occurs in two effectively consecutive steps, one occupying the lag period and the other represented by the sigmoid precipitation curve. Our results show that both steps are accelerated by increasing the temperature. Moreover, they show that fibril width in this closed system is determined during the lag period, i.e. during the first step, and is not affected by a subsequent change of rate of precipitation, at least when temperature is the experimental variable. It would appear therefore that the ultimate fibril width is decided before any appreciable precipitation has taken place. This can only mean that all or most of the fibrils are formed during the lag period. Jackson (1955), in her quantitative study of the formation of collagen fibrils in tissue cultures of embryonic avian tendon, noted that at any stage in their formation there was only a small variation of fibril width in any one bundle of fibrils and suggested that all the fibrils in a bundle must therefore have formed almost simultaneously at a very early stage. These results thus support the view that fibrils form *in vivo* by a similar mechanism to the formation of fibrils *in vitro*.

The width of collagen fibrils observed *in vivo* varies widely from tissue to tissue (Banfield, 1954; Gross, 1948, 1950; Schwartz, 1957). The results suggest that differences of the pH, ionic strength and temperature in the tissue at the time of fibril formation might be significant factors determining this variation.

Opacity measurements (Shulman & Ferry, 1950; Edsall & Lever, 1951) and electron microscopy (Hawn & Porter, 1947; Porter & Hawn, 1949) have been used to study the conversion of fibrinogen into fibrin clots, a system somewhat similar to the precipitation of collagen (Vanamee & Porter, 1951; Schmitt *et al.* 1955*a*; Schmitt, 1957). As in the collagen system, coarseness of the precipitate or clot (wide fibrils and high degree of lateral aggregation of fibrils) is associated with high opacity, and vice versa, and there is no simple correlation of these properties with rate of precipitation.

In view of the fact that the fibrinogen–fibrin conversion involves an enzymic step, however, detailed comparison of the two systems at this stage would not be profitable, for it seems unlikely that a similar enzymic step occurs during the collagen precipitation *in vitro* discussed above.

SUMMARY

1. The precipitation of collagen fibrils in solutions of acid-soluble collagen extracted from calf dermis has been followed turbidimetrically under a range of experimental conditions and the final precipitates have been examined by electron microscopy.

2. All the precipitates consisted of fibrils bearing 640 Å striations and in each precipitate the distribution of fibril width was fairly narrow. Lateral aggregation of the fibrils was most pronounced in those precipitates containing the widest fibrils.

3. When the rate of precipitation was increased by increasing the temperature or lowering the ionic strength, fibril width decreased, but when the rate was increased by reducing the pH, fibril width increased. The variation of rate of precipitation with ionic strength and pH indicates that it is controlled to a significant degree by electrostatic interaction between the soluble collagen particles.

4. All precipitation curves showed a lag period, followed by a sigmoid growth curve. Both phases were accelerated by increase of temperature but the width of the ultimate fibrils was affected only if the temperature was increased during the lag period. It is concluded that most of the fibrils were formed during the lag period.

5. These observations are discussed in relation to the development of collagen fibrils *in vivo*.

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The Formation of Fibrils from Collagen Solutions

2. A MECHANISM OF COLLAGEN-FIBRIL FORMATION

BY G. C. WOOD
Nuffield Gerontological Research Unit, Department of Medicine, University of Leeds

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In the preceding paper (Wood & Keech, 1960) evidence was adduced for the hypothesis that precipitation of collagen fibrils from collagen solutions occurs in two steps, the first occurring during the lag period, the second being represented by the sigmoid precipitation curve. This suggestion has also been put forward by Bensusan & Hoyt (1958). The ultimate fibril width appears to be determined during the first step. It is the purpose of the present paper to see how the experimental results may be interpreted quantitatively in terms of a two-step mechanism.

Freshly prepared collagen precipitates can be redissolved by altering the pH or temperature and then reprecipitated by returning these variables to their original values (Jackson, 1957; Gross, 1958). It seems reasonable therefore to regard the formation of collagen fibrils as precipitation of the protein, in an ordered form, from solution in which it is supersaturated under the particular conditions of pH, ionic strength, temperature, etc. One might expect therefore some similarity between the precipitation of collagen and the precipitation of sparingly soluble inorganic salts, in crystalline form, from supersaturated solution.

The kinetics of the latter process has been widely studied and in many cases it has been recognized that the process takes place in two steps: (*a*) aggregation of the particles in solution to form nuclei, i.e. clusters of particles of just sufficient size to be stable as a separate phase: (*b*) growth of these nuclei, by accretion of soluble material, to

form a visible precipitate. Equations describing the kinetics of these two steps have been derived and applied with some success to a number of systems (e.g. Johnson & O'Rourke, 1954; Duke & Brown, 1954; Nielsen, 1955, 1958; Collins & Leinweber, 1956).

The mechanism of protein precipitation is probably a good deal more complicated than that of inorganic salts. This is likely to be particularly so in the formation of collagen fibrils, where highly asymmetric soluble particles aggregate in a very specific manner with their axes parallel to the ultimate fibril axis (Schmitt, Gross & Highberger, 1955*a*, *b*; Gross, 1956; Schmitt, 1959). Nevertheless, the same general principles should be applicable. Waugh (1957) has, in fact, applied the nucleation-growth concept to the precipitation of insulin fibrils, which, like collagen fibrils, are the result of aggregation of asymmetric particles.

It is of interest therefore to see how far the idea accounts for the rate of formation of collagen fibrils.

Following Waugh's treatment of the problem, the rate of formation of nuclei,

$$\frac{dn}{dt} = k_1 C^x, \tag{1}$$

where C is concentration of collagen, n is concentration of nuclei, x is a constant and k_1 is a rate constant. This equation implies that x collagen particles must come together simultaneously to form a nucleus. The rate of growth of each precipitated particle,

$$\frac{dm}{dt} = k_2 AC, \tag{2}$$

where m is mass of the precipitated particle, A is aggregating area and k_2 is a rate constant. A is assumed to be proportional to the surface area and the further assumption is made that the shape of the precipitated particle remains unchanged during its growth. Then $A \propto m^{\frac{2}{3}}$, and equation (2) becomes

$$\frac{dm}{dt} = k_3 m^{\frac{2}{3}} C. \tag{3}$$

The rate of growth is thus assumed to be controlled by the condensation of the dissolved collagen on to the precipitate and not by its diffusion to the surface of the precipitated particles.

If the growth process is reversible, equation (3) must be modified to

$$\frac{dm}{dt} = k_3 m^{\frac{2}{3}} (C - C_s), \tag{4}$$

where C_s is the value of C at equilibrium, i.e. the solubility of the collagen.

In the very early stages of precipitation, when $C \simeq C_0$ (C_0, initial collagen concentration) equation (4) may be integrated:

$$m = \frac{k_3^3 (C_0 - C_s)\, t^3}{27}. \tag{5}$$

Introducing equation (1),

$$m\,dn = \frac{k_1 k_3^3 C_0^x (C_0 - C_s)^3\, t^3}{27}\,dt,$$

therefore

$$(C_0 - C) = \int_0^{n_t} m\,dn = \frac{k_1 k_3^3 C_0^x (C_0 - C_s)^3\, t^4}{108}.$$

Introducing the extent of precipitation,

$$p = (C_0 - C)/(C_0 - C_s),$$

and putting $(C_0 - C_s)/C_0 = \beta$,

$$p = \frac{K_1 \beta^3 C_0^{(x+2)}}{108}\, t^4, \tag{6}$$

where $K_1 = k_1 k_3^3$.

This relationship will clearly result in an apparent lag period such as is actually observed (Bensusan & Hoyt, 1958; Wood & Keech, 1960).

As the collagen concentration falls appreciably below C_0 the rate of nucleation as given by equation (1) falls rapidly due to the term C^x. At the same time the rate of growth of each precipitated particle as given by equation (4) increases as the particle grows, due to the term $m^{\frac{2}{3}}$, until eventually the fall of collagen concentration slows growth down too. Thus the major part of precipitation will occur by growth of those nuclei formed early in the reaction, i.e. during the lag period. The contribution due to growth of nuclei formed later will be small, its actual extent depending on the relative values of k_1, k_3 and x. Assuming that their contribution is negligible, we have at the end of the lag period a set of small particles whose subsequent growth is responsible for all further precipitation.

It can now be seen how the observed two-step nature of precipitation (Wood & Keech, 1960) arises in terms of this mechanism. The first step, occupying the lag period, consists mainly of the formation of nuclei whereas the second consists of the growth of these nuclei into fibrils. The fibrillar nature of the precipitate results from the asymmetry of the nuclei and of the collagen macromolecules themselves. In the closed system studied experimentally the size of the fibrils is determined by the number and the shape of the nuclei and is hence decided during the lag period. It is not affected by change in rate of growth after the lag period, provided that the manner in which growth takes place does not change at the same time. The mechanism thus accounts qualitatively for the observation that although precipitation is accelerated by increasing the temperature either before or

after the lag period, fibril width is altered only if the temperature is increased before the lag period and is not affected by increasing the temperature after the lag period.

For low values of p the predicted course of precipitation is given by equation (6). The size distribution and hence the growth characteristics, at the end of the lag period, of those particles formed during the lag period might be calculated from equation (5) and the integrated form of equation (1):

$$n = k_1 C_0^x t. \tag{7}$$

The time course of precipitation after the lag period could then be calculated by summing the contribution of all the species of particle. The calculation can be simplified by replacing the distribution of particles by a concentration, n_e, of hypothetical identical particles. The value of n_e and the size of these particles is determined by the conditions that their total surface area and total mass must be the same as those of the actual distribution of particles. Then from equation (4)

$$n_e \frac{\mathrm{d}m}{\mathrm{d}t} = k_3 n_e m^{\frac{2}{3}} C,$$

which may be transformed into

$$\frac{\mathrm{d}p}{\mathrm{d}t} = K_2 \beta^{\frac{2}{3}} C_0^{\frac{2}{3}} p^{\frac{2}{3}} (1-p), \tag{8}$$

where $K_2 = k_3 n_e^{\frac{1}{3}}$. On integration this becomes:

$$K_2 \beta^{\frac{2}{3}} C_0^{\frac{2}{3}} t = \int \frac{\mathrm{d}p}{p^{\frac{2}{3}}(1-p)} = \mathbf{I}, \tag{9}$$

where

$$\mathbf{I} = \tfrac{1}{2} \ln \left\{ \frac{R^2+R+1}{(R-1)^2} \right\} + \sqrt{3} \tan^{-1} \left\{ \frac{\sqrt{3}}{1+2R^{-1}} \right\}$$

and $R = p^{\frac{1}{3}}$.

Application of equations (6) *and* (9) *to experimental data*

The experimental data are those given in the preceding paper (Wood & Keech, 1960). In all the experiments the collagen was almost completely precipitated and therefore $\beta \simeq 1$.

Application of equation (6). Graphs of p against t^4 for a series of experiments at different collagen concentrations are shown in Fig. 1. As predicted by equation (6) these are linear at low values of p. According to equation (6) the slopes (S) are given by

$$\log S = \log \frac{K_1}{108} + (x+2) \log C_0.$$

$\log S$ is plotted against $\log C_0$ in Fig. 2. The linearity of the curve indicates that K_1 and x are independent of collagen concentration and the values of the parameters calculated from the slopes and intercepts of these and other similar graphs are given in Tables 1–3. Thus is appears that equation (6) satisfactorily describes precipitation in the region of low p. The experimental error in measurement of p in this region is, however, quite large so that the experiments do not provide a very critical test and the values of K_1 and x are only approximate.

Application of equation (9). The integral $\mathbf{I}$ is plotted in Fig. 3 as a function of p. By the use of this graph to obtain $\mathbf{I}$ from p, $\mathbf{I}$ was plotted against time (t) for a set of data at different collagen concentrations (Fig. 4). As predicted by equation (9),

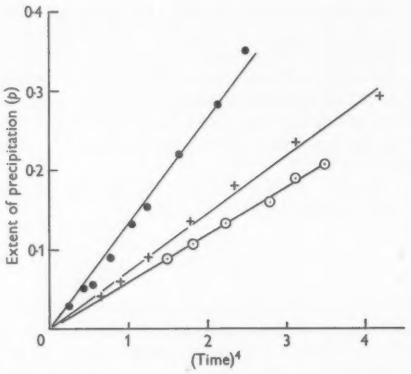

Fig. 1. Early stages of precipitation. Application of equation (6) to experimental data at pH 7·1, I 0·23 and 25°. Initial collagen concn.: ●, 0·084%; +, 0·028%; ⊙, 0·017%. Units of (time)⁴ are: ●, 10^4 min.⁴; +, 10^6 min.⁴; ⊙, 10^7 min.⁴

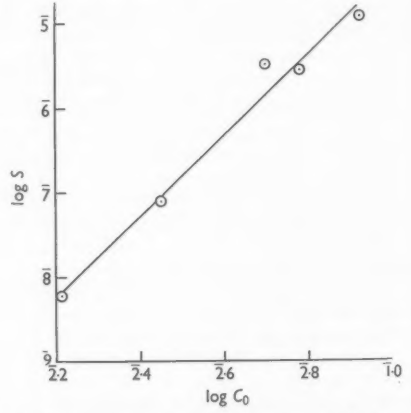

Fig. 2. Early stages of precipitation. Application of equation (6). S is the slope of graph of p against t^4.

the curves are linear over most of their course. The slopes are proportional to K_2, whereas the intercepts, t_c, mark the ends of the lag periods. The deviation from linearity at low values of p might be expected, due to overlap of the nucleation and growth phases, whereas deviation at values of p approaching unity might be due to the sensitivity of **I** to errors in p in this region. In spite of these deviations equation (9) describes the shape of the precipitation curves quite well, as shown in Fig. 5, where the smooth curves were calculated from values of K_2 and t_c obtained from straight lines such as those shown in Fig. 4. Similar results were obtained for a wide range of conditions and the values of K_2 and t_c are given in Tables 1–3.

Table 1. *Effect of collagen concentration on the parameters for nucleation and growth*

Experimental conditions: 25°; pH 7·1; I 0·23. For definitions of C_0, x, K_1, t_c and K_2, see text. The results are for two different preparations of collagen solution.

C_0 (%)	x	K_1	t_c (min.)	K_2
0·084	2·7	227	4·7	1·49
0·061			5·8	1·10
0·050			5·4	1·32
0·028			14·0	0·76
0·017			25·5	0·49
0·050	2·5	100	3·8	0·99
0·034			7·2	0·58
0·017			14·5	0·49

Table 2. *Effect of* pH *and ionic strength on the parameters for nucleation and growth*

Experimental conditions: 25°; C_0 0·05%. For definitions of x, K_1, t_c and K_2, see text. Each set of results was obtained on a different preparation of collagen solution.

pH	I	x	K_1	t_c (min.)	K_2
6·0	0·23	—	—	2·0	4·42
7·1	0·23	—	—	7·0	1·03
7·5	0·23	—	—	7·5	0·96
8·0	0·23	—	—	6·8	1·33
6·0	0·23	2·5	30 500	2·0	6·12
7·1	0·23	2·7	1 400	5·0	1·77
8·0	0·23	3·0	3 100	4·7	2·28
7·0	0·13	—	—	1·0	7·44
7·0	0·23	—	—	2·5	1·18
7·0	0·31	—	—	26·3	0·45
7·0	0·13	1·8	68 000	1·0	9·28
7·0	0·23	1·8	1 400	5·0	1·77
7·0	0·31	3·3	540	10·0	0·81
6·0	0·13	—	—	1·0	9·36
6·0	0·23	—	—	2·0	4·22
6·0	0·31	—	—	1·0	7·22
8·0	0·13	—	—	3·1	4·57
8·0	0·23	—	—	6·8	1·32
8·0	0·31	—	—	27·0	0·48

Table 3. *Effect of temperature on the parameters for nucleation and growth*

Experimental conditions: pH 7; I 0·23; C_0 0·05%. For definitions of x, K_1, K_2 and $(K_2)_g$ see text.

Temp.	x	K_1	K_2	$(K_2)_g$
20°	—	—	0·18	0·17
25	2·5	100	0·99	1·02
30	1·9	440	2·25	1·55
37	1·6	28 000	11·35	4·85

Fibril width

The successful application of equation (9) to precipitation curves and the fact that the mechanism accounts qualitatively for the observation that fibril size is determined during the lag period supports the assumption that only those nuclei formed during the lag period contribute significantly to precipitation.

According to the simple mechanism, at any stage of precipitation these nuclei will have grown into

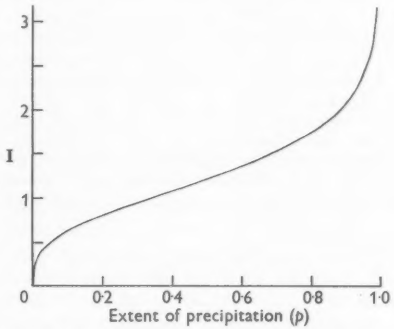

Fig. 3. The integral **I** plotted as a function of p (see equation 9).

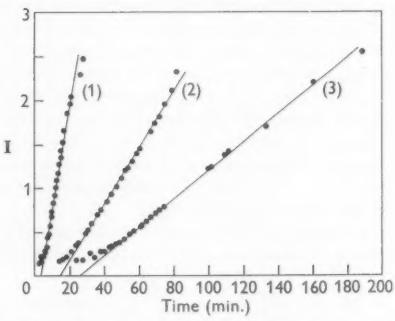

Fig. 4. Precipitation at pH 7·1, I 0·23 and 25° at three different collagen concentrations: (1) 0·084%; (2) 0·028%; (3) 0·017%. Data are plotted in accordance with equation (9).

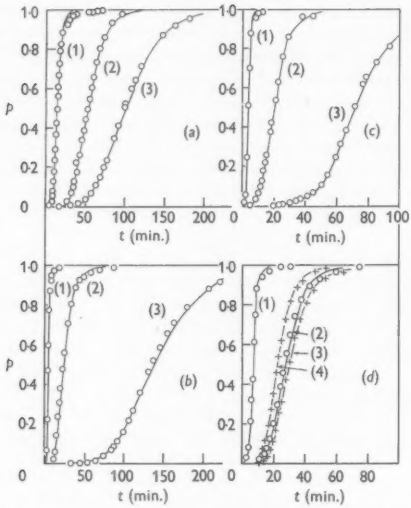

Fig. 5. Precipitation under different experimental conditions. The curves represent the best fit of equation (9) to the experimental data. (*a*) Different collagen concentrations C_0: (1) 0·084%; (2) 0·028%; (3) 0·017%. (*b*) Different temperatures: (1) 37°; (2) 25°; (3) 20°. (*c*) Different ionic strengths, I: (1) 0·13; (2) 0·23; (3) 0·31. (*d*) Different pH values: (1) pH 6·0; (2) pH 7·1; (3) pH 7·5; (4) pH 8·1. Except for the parameters stated in each set, conditions were: pH 7·1; I 0·23; C_0 0·05%; temp. 25°.

fibrils, the largest of which correspond to nuclei formed at zero time and the smallest to nuclei formed at $t = t_c$. From equations (5) and (7), the distribution of fibril width will be such that there are equal numbers of fibrils of different widths, between the two extremes, if, as assumed earlier, the shape of the fibrils remains constant during growth. The observed distribution is narrow but does not conform precisely to this prediction (Wood & Keech, 1960), as may be seen from the histograms reproduced in Figs. 6 and 7. Since it is likely, however, that the shape of the fibrils is distorted during drying for electron microscopy (see Anderson, 1956), this does not necessarily invalidate the assumption of constant shape and, as shown below, the observed distribution is not inconsistent with the mechanism.

From equation (4) the mass of each of the smallest fibrils (m_c) at any time, t, is given approximately by

$$(m_c)_t^{\frac{1}{3}} = \frac{k_3}{3}\int_{t_c}^{t} C\,\mathrm{d}t = \frac{k_3 C_0}{3}\int_{t_c}^{t}(1-p)\,\mathrm{d}t.,$$

Regarding each fibril as a long cylinder of radius r and length l,

$$(m_c)_t = \rho\pi\alpha(r_c)_t^3,$$

where ρ is density, $\alpha(= l/r)$ is a constant and $(r_c)_t$ is radius of each of the smallest fibrils at time t.

$$\text{Therefore} \quad (r_c)_t = \frac{k_3 C_0}{3(\rho\pi\alpha)^{\frac{1}{3}}}\int^{t}(1-p)\,\mathrm{d}t. \tag{10}$$

Similarly, the radius (r_0) of the largest fibrils is

$$(r_0)_t = \frac{k_3 C_0}{3(\rho\pi\alpha)^{\frac{1}{3}}}\int_0^t (1-p)\,\mathrm{d}t = \frac{k_3 C_0}{3(\rho\pi\alpha)^{\frac{1}{3}}}\left\{t_c + \int_{t_c}^{t}(1-p)\,\mathrm{d}t\right\}. \tag{11}$$

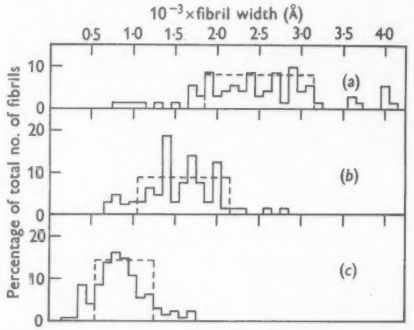

Fig. 6. Comparison of calculated (broken lines) and observed (full lines) fibril-width distributions. Data were obtained at pH 7·1, C_0 0·05% and 25°. Ionic strengths, I: (*a*) 0·31; (*b*) 0·23; (*c*) 0·13.

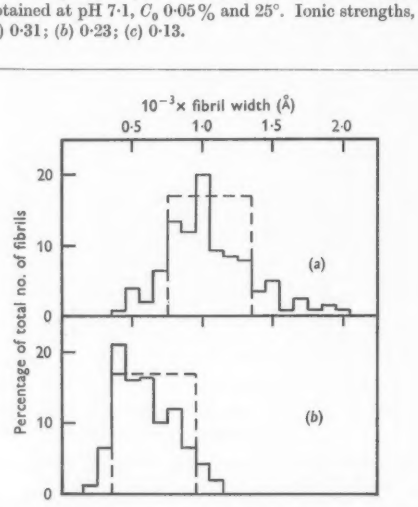

Fig. 7. Comparison of calculated (broken lines) and observed (full lines) fibril-width distributions. Data were obtained at pH 7·1, I 0·23; C_0 0·05%. Temperatures: (*a*) 25°; (*b*) 37°.

When precipitation is complete the ratio of the radii of the largest and smallest fibrils is

$$\frac{(r_0)_\infty}{(r_c)_\infty} = \frac{t_c + \int_{t_c}^{\infty} (1-p)\,dt}{\int_{t_c}^{\infty} (1-p)\,dt}.$$

The right-hand side may be computed from experimental precipitation curves and the results of doing this for several experiments are shown in Table 4. In Figs. 6 and 7 the calculated spread of fibril width is compared with the observed distribution. One limit of the calculated distribution was chosen so that the maximum number of fibrils should be included and in all cases more than 70% of the fibrils come within the predicted limits, indicating that the observed spread of fibril width is consistent with the mechanism.

Separation of the nucleation and growth steps

Each of the parameters K_1 and K_2, resulting from analysis of precipitation curves, involves constants relating to both nucleation and growth. The effect of varying experimental conditions on each of the steps cannot therefore be determined from the variation of K_1 and K_2 alone. The contribution of growth to K_2 can in principle be estimated by analysis of fibril-width distribution, to give n_e, but the existing electron-microscope data are probably not sufficiently precise. Information about the growth step can, however, be obtained by an alternative method. If, in a series of precipitations, nucleation (i.e. lag period) is allowed to proceed under identical conditions in all experiments the effect of any subsequent change of conditions must be attributed to its effect on the growth step.

Table 3 shows data for a series of experiments in which the temperature during nucleation was 25° in all experiments but in which subsequent growth was allowed to proceed at different temperatures. (A typical experiment of this type was described in the preceding paper.)

Table 4. *Effect of ionic strength and temperature on distribution of fibril radius*

Experimental conditions: pH 7·1; C_0 0·05%. For definitions of p, t_c, $(r_0)_\infty$ and $(r_c)_\infty$, see text. Each set of results was obtained on a different preparation of collagen solution.

Temp.	I	$\int_{t_c}^{\infty}(1-p)\,dt$	t_c (min.)	$\frac{(r_0)_\infty}{(r_c)_\infty}$
25°	0·13	1·02	1·0	1·98
25	0·23	5·51	5·0	1·91
25	0·31	26·35	10·0	1·61
25	0·23	16·5	10·0	1·60
37	0·23	1·4	2·0	1·80

Now $K_2 = k_3 n_e^{\frac{1}{2}}$, and since n_e is constant under these conditions

$$\frac{d \ln (K_2)_g}{d\,1/T} = \frac{d \ln k_3}{d\,1/T} = -\frac{E_A}{R},$$

where $(K_2)_g$ is the value of K_2 when nucleation occurs at 25°, E_A is the activation energy for growth, T the absolute temperature and R the gas constant. In introducing E_A it is assumed that the shape of the fibrils and the relation between aggregating area and surface area are independent of the temperature during growth. From the graph of $\log (K_2)_g$ against $1/T$ shown in Fig. 8, E_A = 27 kcal./mole.

Included in Table 3 are data for experiments in which the whole of the precipitations were carried out at different temperatures. Under these conditions:

$$\frac{d \ln K_2}{d\,1/T} = \frac{d \ln k_3}{d\,1/T} + \frac{d \ln n_e^{\frac{1}{2}}}{d\,1/T} = -\frac{E_A}{R} + \frac{d \ln (r_e)_\infty}{d\,1/T}.$$

From the graph of $\log K_2$ against $1/T$ shown in Fig. 8 and the above value of E_A the ratio of the radii of the equivalent fibrils $(r_e)_\infty$ at 25° and 37° is calculated to be 1·6. It can be seen from Fig. 7, which shows the distributions of fibril width at these temperatures, that this figure is of the right order of magnitude.

DISCUSSION

The satisfactory manner in which the simple two-step mechanism accounts for the shape of the precipitation curves and the fact that the predictions about fibril width are consistent with the electron-microscope data strongly suggest that collagen fibrils form *in vitro* by the mechanism outlined above, i.e. by the aggregation of soluble collagen particles to form nuclei, followed by the growth of the nuclei into fibrils.

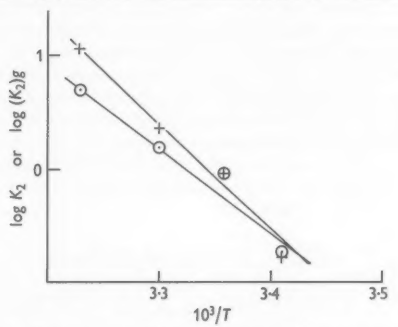

Fig. 8. Variations of K_2 (+) and $(K_2)_g$ (⊙) with absolute temperature, T. For definitions of these parameters see text.

As discussed in the preceding paper, the nature of the soluble collagen particles is uncertain; they may be discrete rod-like collagen molecules or aggregates thereof. The values of the parameter x suggest that two or three of these particles come together to form a nucleus. In view of the asymmetric nature of the collagen molecules the nuclei are probably filamentous; an important point, since the shape as well as the concentration of nuclei plays an important part in determining the rate of precipitation and the size of the ultimate fibrils. The turbidity method used to follow precipitation is, however, unable to give detailed information about nucleation, whose precise nature therefore remains unknown.

The rate of precipitation during the growth phase is determined by the number of fibrils and the rate at which each grows. The rate of the latter process is controlled by the rate of aggregation and by the surface area of the fibrils. Further quantitative electron-microscope observations, particularly at different stages during precipitation, would probably be useful in separating these three factors. It may be noted that the spread of fibril width, as given by equations (10) and (11), should be constant throughout precipitation and this conclusion might also be tested by electron microscopy.

The activation energy for growth under one set of conditions is calculated to be 27 kcal./mole. Bensusan & Hoyt (1958) calculated activation energies for the over-all precipitation process which varied from 23 to 51 kcal./mole according to experimental conditions. The activation energy calculated by their method from the present data is about 40 kcal./mole. If precipitation occurs by the two-step mechanism the precise meaning of Bensusan & Hoyt's activation energies is obscure, since they are calculated from the complex result of two separate processes. The significance which they attach to the fact that their activation energy is independent of ionic strength in formulating a mechanism for precipitation may not therefore be justified. Bensusan & Hoyt observed that the maximum slope of graphs of extinction against time were proportional to the square of the collagen concentration. It may be shown that this result follows from the equations for the two-step mechanism if it is assumed that the final distribution of fibril width is independent of collagen concentration: from equation (8)

$$\left(\frac{\mathrm{d}E}{\mathrm{d}t}\right)_{\max.} = E_\infty \left(\frac{\mathrm{d}p}{\mathrm{d}t}\right)_{\max.} = E_\infty K_2 C_0^{\frac{2}{3}} p_{\max.}^{\frac{2}{3}} (1 - p_{\max.}).$$

It may be shown that $p_{\max.} = 0{\cdot}4$, therefore

$$\left(\frac{\mathrm{d}E}{\mathrm{d}t}\right)_{\max.} = \text{constant} \times E_\infty K_2 C_0^{\frac{2}{3}} = \text{constant} \times \frac{E_\infty k_3 C_0}{(\rho\pi\alpha)^{\frac{1}{3}} (r_e)_\infty}.$$

If $(r_e)_\infty$ and hence E_∞/C_0 are independent of C_0

$$\left(\frac{\mathrm{d}E}{\mathrm{d}t}\right)_{\max.} = \text{constant} \times C_0^2.$$

No electron-microscope data are available to test this but since, as described in the preceding paper, the final extinction (E_∞) of the precipitate is correlated with fibril width, the fact that E_∞/C_0 varies little with collagen concentration supports the assumption.

In deriving the equation for fibril growth it has been assumed that reaction of the soluble collagen particles at the surface of the precipitate is the rate-controlling step. Several workers (Nielsen 1955, 1958, 1959; Frisch & Collins, 1953; Collins & Leinweber, 1956; Ham, 1958) have derived equations for the rate of precipitation when the diffusion of solute to the surface of the precipitate controls the rate and have found that the shape of the precipitation curve is clearly distinguishable from that observed when reaction at the surface is rate-controlling. If, under certain experimental conditions, diffusion of the soluble collagen particles to the surface of the fibril were the rate-controlling step it should be possible to detect this.

SUMMARY

1. Fibril formation is regarded as the result of two processes: (*a*) nucleation, i.e. the aggregation of soluble collagen particles to form nuclei, the smallest aggregates capable of existing as a separate phase; (*b*) growth of the nuclei into fibrils by accretion of further soluble collagen particles. It is assumed that reaction of the soluble collagen particles with the surface of the growing fibrils controls the rate of growth and that the former process is proportional to the surface area of the precipitate and to the collagen concentration.

2. Equations are derived which qualitatively account for the occurrence of a lag period in precipitation, where the nucleation process predominates over growth, and for the observation that the final distribution of fibril width is determined during the lag period.

3. The equation for growth gives a precise description of the shape of precipitation curves over a wide range of conditions. Predictions about fibril width are consistent with experimental observations.

4. The activation energy for growth is calculated to be 27 kcal./mole (at pH 7·1, I 0·23, collagen concentration 0·05 %).

The author wishes to thank Professor R. E. Tunbridge and Dr D. A. Hall for their interest and encouragement throughout this work.

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The Formation of Fibrils from Collagen Solutions

3. EFFECT OF CHONDROITIN SULPHATE AND SOME OTHER NATURALLY OCCURRING POLYANIONS ON THE RATE OF FORMATION

By G. C. WOOD

Nuffield Gerontological Research Unit, Department of Medicine, University of Leeds

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The fact that mucopolysaccharides are abundant in developing connective tissue and the observation that these substances precipitate collagen fibrils from collagen solutions have led to suggestions that they may be important in collagen fibrillogenesis (Meyer, 1946, 1955–56; Gross, 1956).

Except for brief reports by Gross (1956, 1959) and Gross & Kirk (1958) little attention has been paid to the effect of mucopolysaccharides on the kinetics of fibril formation. These workers found that, with two doubtful exceptions, a large number of preparations of chondroitin sulphate, hyaluronic acid, keratosulphate and heparitin sulphate had little or no effect on the rate of fibril formation under the experimental conditions they used. Previous work has shown that the process of collagen-fibril formation may be divided into two phases (Bensusan & Hoyt, 1958; Wood & Keech, 1960), which have been termed nucleation and growth (Wood, 1960). In this paper this work is extended to a study of the effect of the presence of low concentrations of certain mucopolysaccharides in the reaction mixture on the kinetics of fibril formation. Particular attention is paid to the effect of chondroitin sulphate on the overall precipitation process and on the growth step alone. It has been suggested (Wood & Keech, 1960) that different methods of preparing collagen solutions and different methods of pretreating the same collagen solution may result in starting materials, for precipitation experiments, of different degrees of molecular aggregation and therefore having different precipitation properties. A further aim of the present work was therefore to investigate the possibility that the effect of chondroitin sulphate on rate of precipitation depends on the pretreatment of the collagen solution.

The ability to precipitate collagen fibrils is also shown by the nucleic acids (Gross, 1956; Randall, Booth, Burge, Jackson & Kelly, 1955), which, like the mucopolysaccharides, are polyanionic in nature, and the effect of these substances on the rate of fibril formation has therefore also been investigated.

Part of this work has been reported briefly elsewhere (Wood, 1958).

MATERIALS AND METHODS

Collagen solutions. For most of the experiments solutions of collagen (0·1%) in 0·1M-NaCl containing 0·05M-sodium acetate buffer, pH 4·2, were prepared from one sample of purified calf dermis (dermis X), as described previously (Wood & Keech, 1960). For a small number of experiments solutions were prepared in a similar way from another

sample of purified dermis (dermis Y_2). The latter solutions behaved in essentially the same manner as the former but the two differed in one respect. Under the experimental conditions used, collagen from dermis X precipitated completely but only 50–60% of the collagen in solutions from dermis Y_2 precipitated.

Mucopolysaccharides. Chondroitin sulphates prepared from different tissues may be divided into three types. According to Meyer, Linker, Davidson & Hoffman (1956), all are polymers whose repeating units contain *N*-acetyl-D-galactosamine, ester sulphate and hexuronic acid. In chondroitin sulphate A and chondroitin sulphate C the last-named is D-glucuronic acid and in chondroitin sulphate B it is L-iduronic acid. The three substances differ in certain physical properties and in susceptibility to testicular hyaluronidase. Two commercial samples of chondroitin sulphate were used (chondroitin sulphate-1 from Evans Medical Supplies, chondroitin sulphate-2 from L. Light and Co. Ltd.). Both had been prepared from cattle trachea and therefore consisted of chondroitin sulphate A plus a little chondroitin sulphate C (Meyer *et al.* 1956). Samples of chondroitin sulphate A, chondroitin sulphate B and chondroitin sulphate C were kindly supplied by Professor K. Meyer. Heparin and hyaluronic acid were commercial preparations (from British Drug Houses Ltd. and L. Light and Co. Ltd. respectively). Two further, impure, polysaccharide preparations from bovine intervertebral disk were kindly supplied by Dr P. F. Lloyd. Both resulted from deproteinization of aqueous extracts of the tissue. The first (F1) contained predominantly keratosulphate and was similar to fraction A extracted from human nuclei pulposi (Hall, Lloyd, Happey, Horton & Naylor, 1957). The second (F2) contained two components: one had the ionophoretic properties of hyaluronic acid whereas the other was probably a mucoprotein.

Nucleic acids. Two commercial samples of calf-thymus deoxyribonucleic acid (DNA) (DNA-1 from British Drug Houses Ltd., DNA-2 from California Foundation for Biochemical Research) and high-molecular-weight preparations from calf thymus and rat liver (DNA-3 and DNA-4 respectively) were used. Two commercial samples of ribonucleic acid (RNA) (RNA-1 from British Drug Houses Ltd., RNA-2 from Nutritional Biochemicals Corp.) and high-molecular-weight preparations from rat liver and ox liver (RNA-3 and RNA-4 respectively) were employed.

Analytical methods. In addition to the methods described previously (Wood & Keech, 1960) the procedure of di Ferrante (1956) was used to estimate low concentrations of chondroitin sulphate and heparin.

Procedure for studying the effect of the polyanions on the rate of precipitation of collagen. The polyanion was incorporated into the phosphate buffer added to the collagen solution at zero time and the course of precipitation followed turbidimetrically. The composition of the various solutions and the experimental procedure were as described previously (Wood & Keech, 1960).

EXPERIMENTS AND RESULTS

Effect of different polyanions on the overall precipitation

Fig. 1 (*a*) shows the effect of chondroitin sulphate A and heparin on the rate of precipitation at pH 7·1, I 0·23 and 25°. Thus chondroitin sulphate A, at a concentration one-tenth of that of the collagen, accelerated precipitation whereas heparin, at a concentration one-hundredth of that of the collagen, retarded it. The general shape of the precipitation curve was not altered by the presence of either mucopolysaccharide but the extinction attained different final values (E_∞) in each case. Since in all three cases the collagen was completely precipitated the extent of precipitation is given by $p = E/E_\infty$.

It has been shown by Wood (1960) that the sigmoid sections of precipitation curves obtained in the absence of added polyanion are closely described by equations of the form:

$$\frac{dp}{dt} = K_2 C_0^{\frac{1}{2}} p^{\frac{1}{2}}(1-p), \qquad (1)$$

where C_0 is the initial collagen concentration, p is the extent of precipitation and K_2 is a constant. The smooth curves shown in Fig. 1 (*b*) represent the best fit of equation (1) to the data, indicating that it also provides a satisfactory description of the results when these polyanions are added. Similar

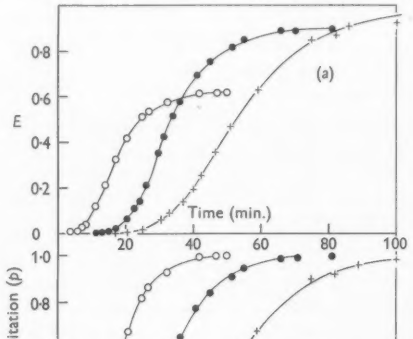

Fig. 1. Effect of chondroitin sulphate A and heparin on the rate of precipitation of collagen at pH 7·1, I 0·23, 25° and with collagen 0·05%. Reaction mixtures contained: ○, 0·005% of chondroitin sulphate A; +, 0·0005% of heparin; ●, no added polysaccharide (control). (*a*) Time course of change of E. (*b*) Time course of extent of precipitation ($p = E/E_\infty$, where E_∞ is final value of E); the curves represent the best fit of equation (1) (see text) to the experimental data.

Table 1. *Effect of chondroitin sulphate and heparin on the rate of precipitation of collagen*

Experimental conditions: pH 7·1; I 0·23; temp. 25°; collagen 0·05%; polysaccharide 0·005%, except in one experiment with heparin where the concentration was as indicated (0·0005%). For definition of t_c, K_2 and E_∞ see text. Each set of data was obtained with a different preparation of collagen solution. Since the protein in the collagen solution was about 93% of collagen (Wood & Keech, 1960) the data in column 4 indicate complete precipitation of the collagen.

Polysaccharide	t_c (min.)	K_2	Extent of precipitation of protein (%)	E_∞
—*	10·0	0·93	—	0·90
Chondroitin sulphate A*	1·5	1·28	—	0·62
Heparin (0·0005%)*	16·0	0·59	—	1·00
Heparin†	> 60	—	89	—
—	7·7	1·17	92	0·61
Chondroitin sulphate A	0·2	2·34	92	0·53
	0·8	1·68	—	0·54
Chondroitin sulphate B	10·3	1·02	94	0·49
	12·5	0·88	—	0·56
Chondroitin sulphate C	1·2	2·05	92	0·50
	1·8	2·17	—	0·49
—	7·5	1·17	92	0·63
Chondroitin sulphate-1	0·5	2·27	93	0·50
Chondroitin sulphate-2	0·9	2·20	94	0·59

* Data for curves shown in Fig. 1.

† In this experiment the precipitate was flocculent and rose to the top of the cell so that the precipitation curve could not be determined turbidimetrically.

Table 2. *Effect of mucopolysaccharides on the rate of precipitation of collagen*

Experimental conditions: pH 7·1; I 0·23; temp. 25°; collagen 0·05%; polysaccharide 0·005%. For definitions of t_c, K_2 and E_∞ see text. As in Table 1, column 4 indicates complete precipitation of collagen.

Polysaccharide	t_c (min.)	K_2	Extent of precipitation of protein (%)	E_∞
—	7·3	1·17	92	0·63
Hyaluronic acid	5·1	1·25	93	0·71
	8·2	1·03	—	0·70
F1	1·4	1·32	94	0·62
	3·3	1·32	—	0·50
F2	3·6	0·81	92	0·70

results were obtained with other polyanions, whose effect may therefore be conveniently stated in terms of K_2, E_∞ and the lag period t_c. The parameter K_2 is, from equation (1), proportional to the slope of the graph of p against t for a given value of p.

As shown in Table 1, the accelerating effect of chondroitin sulphate A is indicated by decrease of t_c and increase of K_2, whereas the retarding effect of heparin is shown by increase of t_c and decrease of K_2. Chondroitin sulphate-1, chondroitin sulphate-2 and chondroitin sulphate C also accelerated precipitation but chondroitin sulphate B did not have a significant effect on K_2, although it increased t_c slightly. Table 2 shows that F1 (keratosulphate) accelerated precipitation in a similar way to chondroitin sulphate A and chondroitin sulphate C [in Wood (1958) keratosulphate was erroneously

Table 3. *Effect of nucleic acids on the rate of precipitation of collagen*

Experimental conditions: pH 7·1; I 0·23; temp. 25°; collagen 0·05%; nucleic acid 0·005%. Each set of results was obtained with a different preparation of collagen solution from dermis X. For definitions of t_c and K_2 see text.

Nucleic acid	t_c (min.)	K_2
—	6·0	2·05
DNA-1	12·0	1·32
DNA-2*	> 60	—
DNA-3	11·2	0·66
DNA-4	10·5	0·66
—	5·0	1·61
RNA-1	7·2	1·76
RNA-2	6·1	1·61
RNA-3	9·8	0·88
RNA-4	8·8	1·83

* DNA-2 gave a flocculent precipitate on which turbidity measurements could not be made (cf. heparin, Table 1).

stated to retard precipitation] whereas hyaluronic acid and F2 did not have a marked effect.

When the nucleic acids were studied the outstanding feature of the results was the marked retarding effect of all the samples of DNA, the magnitude of which varied from one preparation of DNA to another (Table 3). All the preparations of RNA increased t_c slightly. Three of them had little effect on K_2 but RNA-3 decreases it. The effect of DNA-3 and RNA-3 on the rate of precipitation in collagen solutions prepared from dermis Y_2 is shown in Table 4. DNA-3 retarded precipitation even when its concentration was as low as 0·005%. RNA-3 increased t_c slightly but had no significant effect on K_2.

The variation of rate of precipitation from one preparation of collagen solution to another, noted previously (Wood & Keech, 1960), is also apparent in the data for the control experiments in Tables 1–4. The magnitude of the effect of the different polyanions also varied appreciably from one experiment to another but there is no doubt that under these conditions chondroitin sulphate A, chondroitin sulphate C and F1 (keratosulphate) accelerated precipitation and that DNA and heparin retarded it.

Tables 1 and 2 show that in spite of the accelerating or retarding effect of any of the mucopolysaccharides the collagen was completely precipitated. The variation of E_∞ must therefore be due to the effect of these substances on the structure of the precipitates. Those mucopolysaccharides which accelerated precipitation (chondroitin sulphate A, chondroitin sulphate C and F1) caused E_∞ to be lower than in control experiments, whereas heparin, which retarded precipitation, increased it. Chondroitin sulphate B also lowered E_∞ and hyaluronic acid and F2 increased it, although these three compounds had little effect on rate of precipitation.

Effect of chondroitin sulphate on the overall precipitation at different values of pH *and ionic strength*

The effect of chondroitin sulphate-1 at four values of pH (I 0·23) and three values of I (pH 7·1) is shown in Table 5. The effect of these variables in the absence of chondroitin sulphate-1 (controls) agrees with that described previously (Wood & Keech, 1960). The shortening of the lag period by chondroitin sulphate-1 was not affected appreciably by changing pH but became more pronounced on increasing I. The increase of K_2 caused by chondroitin sulphate was enhanced by lowering the pH from 8 to 6 and by increasing I. In those experiments where the accelerating effect of chondroitin sulphate-1 was most marked E_∞ was lower than in the controls, but at pH 8, I 0·23 and at pH 7, I 0·13, E_∞ was higher when chondroitin sulphate-1 was present.

Effect of chondroitin sulphate-1 on the growth step of precipitation

Fig. 2 shows the results of three experiments designed to test the effect of chondroitin sulphate-1 on the growth step. In each experiment four

Table 4. *Effect of different concentrations of RNA-3 and DNA-3 on the rate of precipitation of collagen*

Experimental conditions: pH 7·1; I 0·23; temp. 25°; collagen 0·05%. Each set of results was obtained with a different preparation of collagen solution from dermis Y_2. For definitions of t_c and K_2, see text.

Nucleic acid	Concn. (%)	t_c (min.)	K_2
DNA 3	—	6·5	1·04
	0·0005	9·7	0·81
	0·0025	11·2	0·66
	0·0050	41·0	0·37
RNA 3	—	7·0	0·96
	0·0005	11·6	0·88
	0·0025	11·6	0·88
	0·0050	8·8	0·96

Table 5. *Effect of chondroitin sulphate-1 on the rate of precipitation of collagen at different values of* pH *and ionic strength*

Collagen 0·05%. Chondroitin sulphate-1 0·005%. Temp. 25°. Each set of results was obtained with a different preparation of collagen solution. For definitions of t_c, K_2 and E_∞ see text.

		t_c (min.)		K_2		E_∞		Percentage change due to chondroitin sulphate		
pH	I	Control	Chondroitin sulphate	Control	Chondroitin sulphate	Control	Chondroitin sulphate	t_c	K_2	E_∞
6·0	0·23	2·0	0·5	4·42	10·08	0·94	0·50	−75	+129	−47
7·1	0·23	7·0	1·0	1·03	2·05	0·83	0·73	−86	+99	−12
7·5	0·23	7·5	1·5	0·96	1·85	0·70	0·72	−80	+93	+3
8·0	0·23	6·8	1·5	1·33	1·68	0·46	0·73	−78	+26	+59
7·1	0·13	1·0	1·0	7·44	7·44	0·46	0·61	0	0	+33
7·1	0·23	2·5	1·7	1·18	1·39	1·08	1·00	−32	+18	−8
7·1	0·31	25·3	1·0	0·45	1·07	1·10	0·91	−96	+138	−17

samples of collagen solution (1·5 ml.) were equilibrated at 25°. On two of these, precipitation runs were done by the usual procedure, one with chondroitin sulphate present (curve 2) and one without (curve 1). To each of the remaining two samples 1·55 ml. of buffer was added at zero time and the mixtures were allowed to stand at 25° for the duration of the lag period, determined from curve 1. To one of them 0·15 ml. of chondroitin sulphate-1 (1% in 0·1M-NaCl) was added with stirring and to the other 0·15 ml. of 0·1M-NaCl. The course of subsequent precipitation is given by curves 3 and 4 respectively. All the reaction mixtures had identical volume and salt composition after the lag period.

Comparison of curves 1 and 2 in each set indicates the accelerating effect of chondroitin sulphate-1 on the over-all process (nucleation + growth) described above. Comparison of curves (3) and (4) indicates that when chondroitin sulphate-1 was added at the end of the lag period at pH 6 and 7 (I 0·23), it had no appreciable effect either on the rate of growth or on E_∞, and that at pH 7, I 0·31 it retarded growth slightly. Comparison of curves (1) and (4) shows that the slight difference in the composition of the reaction mixtures during the lag period between (1) and (2) on the one hand and (3) and (4) on the other, together with the stirring of (3) and (4) at the end of the lag period, had little effect on subsequent precipitation. It is concluded that, under these conditions, the acceleration of the over-all precipitation by chondroitin sulphate-1 and its effect on E_∞ is due mainly, if not wholly, to its action during the lag period.

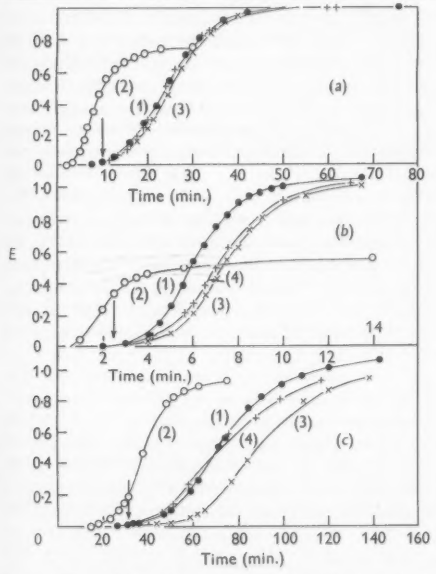

Fig. 2. Effect of chondroitin sulphate-1 on the growth step of precipitation. Collagen concn. 0·05%; chondroitin sulphate concn. 0·005%; temp. 25°. (*a*) I 0·23, pH 7·1; (*b*) I 0·23, pH 6·0; (*c*) I 0·31, pH 7·1. In (*a*), (*b*) and (*c*): (1) chondroitin sulphate absent; (2) chondroitin sulphate present throughout precipitation; (3) chondroitin sulphate absent during lag period, the end of which is indicated by the arrow; (4) chondroitin sulphate absent, mixture stirred at end of lag period as in (3). In (*a*) curve (4) coincides with curve (1).

Effect of chondroitin sulphate-1 on the rate of precipitation from collagen solutions pretreated with phosphate buffer, pH 7

The following experiments (Fig. 3) were done to investigate the possibility that the effect of chondroitin sulphate on the kinetics of fibril formation depends on the pretreatment of the collagen solution. In each experiment 1·55 ml. of buffer, pH 7·1, cooled to 0°, was added to each of

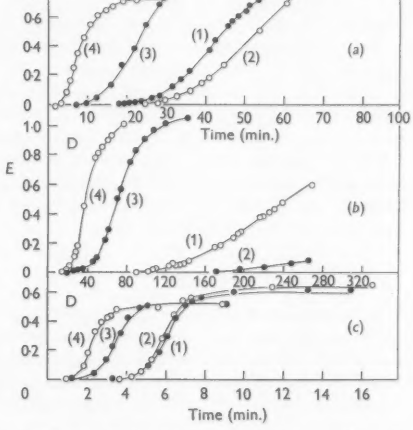

Fig. 3. Effect of chondroitin sulphate-1 on the rate of precipitation of collagen from collagen solutions previously kept at pH 7·0 for 16 hr. at 0°. Collagen concn. 0·05%; chondroitin sulphate concn. 0·005%; pH 7·0. (*a*) I 0·23, 25°; (*b*) I 0·31, 25°; (*c*) I 0·31, 37°. In (*a*), (*b*) and (*c*): (1) chondroitin sulphate absent; (2) chondroitin sulphate present. For comparison curves (3) and (4) show the effect of chondroitin sulphate on rate of precipitation from collagen solutions not previously adjusted to pH 7·0: (3) chondroitin sulphate absent; (4) chondroitin sulphate present. The collagen concentration and salt composition of the medium were the same in all four experiments.

two samples of collagen solution (1·15 ml.) at 0° and the mixtures were allowed to stand at 0° for 16 hr. To one, 0·15 ml. of 0·1M-NaCl was added with stirring, and to the other, 0·15 ml. of 1% chondroitin sulphate-1 in 0·1M-NaCl was added. The temperature was rapidly raised to 25° or 37° and precipitation followed (curves 1 and 2 respectively). Thus when, as described above, the pH of the collagen solution was first brought to neutrality and precipitation was then initiated by raising the temperature, chondroitin sulphate-1 retarded precipitation at 25° and had no significant effect at 37°. On the other hand, when, as in preceding experiments, the temperature was raised first and precipitation initiated by adjusting pH (curves 3 and 4), chondroitin sulphate-1 accelerated precipitation at both 25° and 37°.

Previous adjustment of the pH to neutrality had itself a marked retarding effect on precipitation (compare curves 1 and 3). The magnitude of this effect is too great to be accounted for merely by the time required to raise the temperature from 0° to 25° or 37°.

Incorporation of mucopolysaccharides into the collagen precipitates

Precipitates were prepared in the presence of different concentrations of chondroitin sulphate-1 or heparin, at pH 7·1, I 0·23, by allowing the reaction mixtures to stand at 25° for 16 hr. The precipitates were coagulated by stirring, centrifuged and the concentration of mucopolysaccharide in the supernatant was estimated. Little, if any, chondroitin sulphate-1 was incorporated into the precipitate (Table 6). An appreciable fraction of the added heparin was retained in the precipitate but the ratio of heparin to collagen in the precipitate was, nevertheless, low.

DISCUSSION

The results show that low concentrations of some mucopolysaccharides and nucleic acids have a marked effect, under certain experimental conditions, on the rate of precipitation of collagen and on the final extinction (E_∞) of the precipitate, but do not affect the amount of collagen ultimately precipitated.

In a parallel electron-microscope study M. K. Keech (in preparation) has shown that precipitates prepared at pH 7, I 0·23, in the presence of the mucopolysaccharides and nucleic acids used in the present work, were in the form of fibrils bearing the 640 Å striations characteristic of native collagen. When those polyanions which lower E_∞ relative to the controls at pH 7, I 0·23 (chondroitin sulphate-1, chondroitin sulphate-2, chondroitin sulphate A, chondroitin sulphate B and keratosulphate) were present during precipitation the fibrils were thinner than in controls and when those were present which increase E_∞ (heparin, hyaluronic acid and DNA) the fibrils were thicker than in controls. Keech's results thus indicate that there is a qualitative correlation between E_∞ and fibril width similar to that observed previously when precipitation of collagen in the absence of added polyanion was studied (Wood & Keech, 1960).

The polyanions which accelerate precipitation at pH 7, I 0·23 (chondroitin sulphate-1, chondroitin sulphate-2, chondroitin sulphate A, chondroitin sulphate C and keratosulphate) decrease E_∞ and fibril width, whereas those which retard precipitation (heparin, DNA) increase E_∞ and fibril width. Chondroitin sulphate B and hyaluronic acid, however, which have no appreciable effect on rate of precipitation under these conditions, decrease and increase respectively E_∞ and fibril width. In addition, chondroitin sulphate-1 acting at higher pH still accelerates precipitation but increases E_∞ and therefore, presumably, fibril width. Thus the lack of simple correlation between rate of precipitation and fibril width observed at different ionic strength and pH (Wood & Keech, 1960) is also found when rate of fibril formation is altered by the polyanions.

It was concluded from earlier work that precipitation is the result of two effectively consecutive processes, one occurring during the lag period (nucleation) and the other during the growth phase (growth of nuclei into fibrils) (Wood, 1960). Since, in spite of the presence of the polyanions in the reaction mixtures, all the precipitation curves

Table 6. *Incorporation of chondroitin sulphate and heparin into collagen precipitates*

Experimental conditions: pH 7·1; I 0·23; temp. 25°; collagen 0·05%.

Polysaccharide	Concn. of polysaccharide in reaction mixture (%)	Polysaccharide remaining in precipitate (%)	Ratio (w/w) polysaccharide / collagen in precipitate
Chondroitin sulphate-1	0·0055	4	0·004
	0·00275	2	0·002
Heparin	0·0047	16	0·015
	0·00235	24	0·011
	0·00094	45	0·008

are described by equation (1), it is concluded that these substances do not alter the way in which the soluble collagen particles aggregate during growth. This conclusion is supported by the observation that all the precipitates consisted of fibrils with 640 Å striations.

Further evidence for the two-step nature of fibril formation is given by the observation that chondroitin sulphate-1 accelerates precipitation and affects fibril width only if it is present during the lag period. This is another demonstration that the ultimate fibril width is determined in the very early stages of precipitation, and, if the two-step mechanism is correct, must mean that chondroitin sulphate-1 accelerates precipitation by affecting nucleation. In view of this the fact that chondroitin sulphate-1 did not accelerate precipitation (at pH 7, I 0·23 and 25°), when the collagen solution had previously been adjusted to pH 7 for 16 hr. at 0°, suggests that in this experiment nucleation had occurred before chondroitin sulphate-1 was added, i.e. during the period at pH 7 and 0°. The occurrence of nucleation before addition of chondroitin sulphate may well account for Gross's observation (Gross, 1956, 1959; Gross & Kirk, 1958), confirmed in the present work, that a number of preparations of chondroitin sulphate did not accelerate precipitation at 37°, for his experimental procedure involved prior dialysis of the collagen solutions against cold neutral phosphate buffer.

Some indication of the possible mode of action of chondroitin sulphate and the other polyanions may be gained by further consideration of the two-step mechanism of fibril formation. According to this, nucleation may be affected in two ways. First, chondroitin sulphate might accelerate nucleation, increase the number of nuclei formed and hence accelerate subsequent precipitation and decrease the size of the ultimate fibrils. Secondly, it might alter the shape of the nuclei formed. Since the nuclei are assumed to grow at a rate proportional to their surface area, increase in the axial ratio of the nuclei must cause an increase in their rate of growth as well as a decrease in the width of the fibrils produced. Since chondroitin sulphate-1 is able both to accelerate precipitation by acting during the period of nucleation and to retard it when present only during the growth step (see Fig. 2c), the first possibility, acceleration of nucleation, is therefore likely only if nucleation and growth are fundamentally different processes. In order to achieve the increase of axial ratio of the nuclei, required by the alternative mechanism, chondroitin sulphate-1 would have to promote end-to-end aggregation of the soluble collagen particles. It would appear, from the marked dependence of the effect of chondroitin sulphate-1 on the pH and ionic strength of the medium, that electrostatic intermolecular forces play an important part. It has been suggested (Kühn, Grassmann & Hofmann, 1957; Kühn, Hofmann & Grassman, 1959; Hodge & Schmitt, 1958) that amino acids with basic side chains (arginine, lysine) are concentrated in the end regions of the soluble collagen macromolecules, and it is suggested that a chondroitin sulphate molecule might promote end-to-end aggregation by the interaction of its negatively-charged sulphate groups with the positively-charged groups of two collagen macromolecules. Only a very small fraction of the added chondroitin sulphate-1 is incorporated into the collagen precipitate, but, since the nuclei constitute only a very small fraction of the fibrils into which they grow, it is possible that chondroitin sulphate constitutes an appreciable fraction of each nucleus.

The retardation of fibril growth by chondroitin sulphate-1, and the even more pronounced retardation by DNA and heparin, might be due to the formation of collagen–polyanion complexes which, because of the high negative charge conferred by the polyanion, aggregate less readily than free collagen particles. This complex formation would have to be reversible since only little polyanion is incorporated into the precipitate although the collagen is completely precipitated.

The mechanisms suggested above, particularly that with reference to the shape of the nuclei, would be expected to have rather specific requirements of the chemical structure, size, shape and charge distribution of the polyanion molecules and this is consistent with the diversity of the effects of the various mucopolysaccharides and nucleic acids. Even such closely related compounds as chondrotin sulphate A and chondroitin sulphate C on the one hand and chondroitin sulphate B on the other have different effects on rate of precipitation.

It is well known that the presence of acid mucopolysaccharides in connective tissues, as indicated by metachromasia and hexosamine determinations, is particularly noticeable in the early stages of collagen-fibril formation (Dunphy & Udupa, 1955; Jackson, 1955). The present work suggests that these mucopolysaccharides might be concerned with regulating nucleation and growth and hence help to determine the rate of formation of fibrils and their ultimate size. Differences in mucopolysaccharide content of different tissues might be connected in this way with variation of fibril width from tissue to tissue; pronounced metachromasia is associated with small fibril diameter in certain tissues (Van den Hooff, 1957).

The interest in DNA arose in the present work from its polyanionic nature. It does not usually occur extracellularly and the observations of its

effect on fibril formation *in vitro* does not at the moment appear to have any physiological implications.

SUMMARY

1. Low concentrations of heparin and deoxyribonucleic acid have been found to retard fibril formation in solutions of acid-soluble collagen at pH 7, I 0·23 and 25°. Two types of chondroitin sulphate (A and C) and a preparation rich in keratosulphate accelerated fibril formation. Another type of chondroitin sulphate (B) and hyaluronic acid had no effect. Those polyanions which accelerated precipitation reduced fibril width and those which retarded precipitation increased it. The accelerating effect of chondroitin sulphate A increased with decreasing pH in the range pH 6–8 and with increasing I in the range 0·13–0·31.

2. Chondroitin sulphate A accelerated precipitation only when present during the lag period. This observation supports the conclusion from earlier work that collagen fibrils form as the result of two consecutive processes, nucleation and growth, and it is concluded that chondroitin sulphate A accelerates precipitation by affecting nucleation. Only a very small proportion of the added chondroitin sulphate was incorporated into the collagen precipitate.

3. When collagen solutions were pretreated with phosphate buffer, pH 7, at 0° addition of chondroitin sulphate A retarded subsequent precipitation at 25°, indicating that nucleation had taken place during pretreatment and that the observed retardation was due to the effect of chondroitin sulphate A on growth.

4. The mode of action of the polyanions is considered in the light of the two-step mechanism of fibril formation and the findings are discussed in relation to the possible role of mucopolysaccharides in the formation of collagen fibrils *in vivo*.

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Steroid Sulphatase, Arylsulphatase and β-Glucuronidase in the Mollusca

BY Y. ANDRÉE LEON, R. D. BULBROOK AND E. D. S. CORNER
Division of Chemistry and Biochemistry, Imperial Cancer Research Fund, London, W.C. 2 and The Plymouth Laboratory of the Marine Biological Association of the United Kingdom

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In recent years considerable interest has been shown in the use of enzymes from molluscan tissues for the hydrolysis of urinary steroid conjugates (Henry & Thevenet, 1952; Stitch & Halkerston, 1953*a*, *b*, 1956; Jayle & Baulieu, 1954). Although these tissues are known to be rich sources of β-glucuronidase and arylsulphatase (Dodgson, Lewis & Spencer, 1953) and to possess steroid sulphatases, the specificity of the latter has been investigated in only two species. An enzyme

present in extracts of *Otala punctata*, a tropical terrestrial gastropod, was reported by Savard, Bagnoli & Dorfman (1954) to hydrolyse dehydro-*epi*androsterone sulphate but not the sulphates of androsterone or testosterone. Roy (1954, 1956*a*) prepared a highly active concentrate of steroid sulphatase from an acetone-dried powder of *Patella vulgata* and showed that the enzyme was specific for the 3β-sulphates of the 5α and Δ^5 steroids, but had no action on the other isomeric 3-sulphates.

The specificity of the steroid sulphatases from these two gastropods limits their usefulness in the hydrolysis of urinary steroid conjugates because both 3α-hydroxy-5α and -5β steroids may be excreted as sulphates. On the other hand, hydrolysis of conjugates with strong acid at higher temperatures leads to the formation of artifacts from some steroids, and the use of milder conditions, such as continuous ether extraction at pH 1, is time-consuming. These factors prompted a search for a convenient source of enzyme capable of hydrolysing androsterone sulphate, and the present study has been primarily concerned with an investigation of the steroid sulphatases present in various marine molluscs. Because many of the animals used in this work had not been investigated as possible sources of arylsulphatase and β-glucuronidase, it was considered worth while to determine whether these enzymes could be detected in each of the species examined, and whether their activities were in any way correlated with those of the steroid sulphatases.

MATERIALS AND METHODS

Extraction of enzymes from the test animals

Most of the species were collected near Plymouth Sound. They were dissected as soon as they had been brought into the laboratory and were used for the preparation of an acetone-dried powder, of the kind which Dodgson & Spencer (1953) have described as powder A. Usually the tissue chosen for study was the digestive gland (hepatopancreas); when the test material was too small for this tissue to be dissected, whole animals, including their shells, were ground in a pestle and mortar and the debris obtained was used in the preparation of powder A.

Substrates

p-Chlorophenyl glucuronide monohydrate was prepared by the method of Spencer & Williams (1951), dipotassium 2-hydroxy-5-nitrophenyl sulphate by the method of Roy (1956*a*), and steroid sulphates by the method of Roy (1956*a*). A pure sample of *p*-nitrophenyl sulphate was obtained from Sigma Chemical Company, Missouri, U.S.A.

Measurement of enzymic activity

β-Glucuronidase. This was assayed by the method of Dodgson *et al.* (1953), in 0·5 M-sodium acetate–acetic acid buffer, pH 4. The pH optimum of 4·0 reported by these workers for the hydrolysis of *p*-chlorophenyl glucuronide with tissue homogenates of *P. vulgata* and *Littorina littorea* was used throughout, but the optimum substrate concentration for the powder prepared from the visceral hump of *P. vulgata* was redetermined because an acetone-dried powder was used instead of a homogenate. As only a small increase in enzymic activity was detected with substrate concentrations greater than 0·045 M (Fig. 1), this concentration of *p*-chlorophenyl glucuronide was used in all the assays of β-glucuronidase. All the estimations of enzymic activity were made with a final concentration of 400 μg. of the powder obtained from each species/ml.

Arylsulphatase. This was assayed with 2-hydroxy-5-nitrophenyl sulphate as the substrate by the method of Dodgson & Spencer (1953), as modified by Dodgson *et al.* (1953). The pH optimum for the hydrolysis of 2-hydroxy-5-

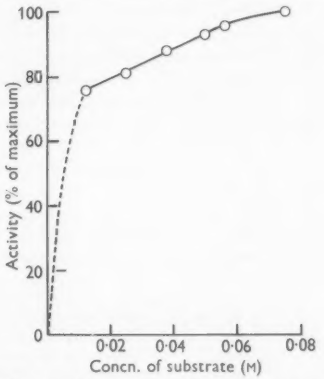

Fig. 1. Effect of substrate concentration on β-glucuronidase activity of crude powder from *P. vulgata*. The volume of the reaction mixture was 0·8 ml., comprising 0·4 ml. of enzyme solution in 0·5 M-acetate buffer, pH 4·0, and 0·4 ml. of *p*-chlorophenyl glucuronide solutions of various concentrations in the same buffer. Incubation was for 1 hr. at 37·5°.

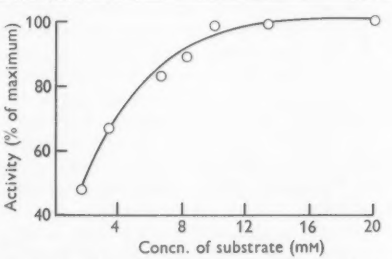

Fig. 2. Effect of substrate concentration on arylsulphatase activity of crude powder from *P. vulgata*. The volume of the reaction mixture was 1·2 ml., comprising 0·6 ml. of enzyme solution in 0·5 M-acetate buffer, pH 5·5, and 0·6 ml. of nitrocatechol sulphate solutions of various concentrations in the same buffer. Incubation was for 1 hr. at 37·5°.

nitrophenyl sulphate with the arylsulphatase of *P. vulgata* has been reported by Roy (1956*a*) to be 5·4–5·6, and a pH of 5·5 was therefore used in the present study. As the enzyme prepared by Roy was highly purified, it was necessary to determine the optimum substrate concentration for the crude powder from *P. vulgata*. At pH 5·5, in acetate buffer, this concentration was found to be 12 mM (Fig. 2). Measurements of enzymic activity were made on 100 μg. of the powder obtained from each species/ml.

Assays of enzymic activity were also carried out with *p*-nitrophenyl sulphate as the substrate and the method used was similar to that described above, except that the liberated *p*-nitrophenol was read at 400 mμ. The pH optimum for the powder prepared from *P. vulgata* was found to be 5·5–5·7 (Fig. 3) and, at pH 5·5, the optimum substrate concentration was 0·03 M (Fig. 4). The assays were carried out in 0·5 M-sodium acetate–acetic acid buffer, pH 5·5.

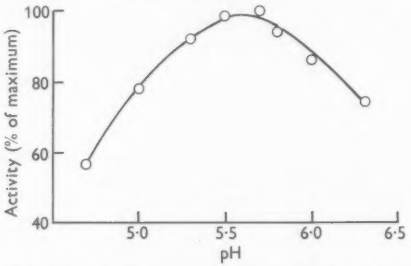

Fig. 3. Effect of pH on arylsulphatase activity of crude powder from *P. vulgata*. The volume of the reaction mixture was 1·2 ml. comprising 0·3 ml., of enzyme solution, 0·3 ml. of 2·0 M-acetate buffers of various pH values, and 0·6 ml. of 0·06 M-*p*-nitrophenyl sulphate. Incubation was for 1 hr. at 37·5°.

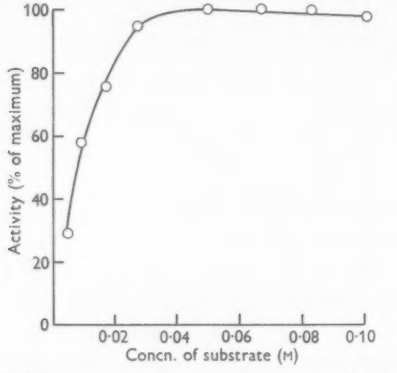

Fig. 4. Effect of substrate concentration on arylsulphatase activity of crude powder from *P. vulgata*. The volume of the reaction mixture was 1·2 ml., comprising 0·6 ml. of enzyme solution in 0·5 M-acetate buffer, pH 5·5, and 0·6 ml. *p*-nitrophenyl sulphate solutions of various concentrations in the same buffer. Incubation was for 1 hr. at 37·5°.

Steroid sulphatase. This was assayed by the method of Roy (1956*a*) with the diluted methylene-blue reagent described by Roy (1956*b*). The absorption of the steroid sulphate–methylene blue complex was measured at 655 mμ (Unicam SP. 400 spectrophotometer). Each enzyme preparation was tested at six different pH values in 0·5 M-sodium citrate–HCl buffer (pH 3·0), 0·5 M-sodium acetate–acetic acid buffers (pH 4·0, 5·0 and 6·0) and 0·5 M-2-amino-2-hydroxymethylpropane-1:3-diol–acetic acid buffers at pH 7·0 and 8·0. The final concentration of the buffers in the assays was 0·125 M after addition of the substrate. The partition of the steroid sulphate–methylene blue complex between the aqueous and chloroform phases was not influenced by pH in the range 4·0–8·0, but at pH 3·0 it increased by about 5% in favour of the chloroform phase. Two controls were therefore used in each experiment, one at pH 3·0 and the second at one of the other pH values. A typical example of the effect of pH on the activity of steroid sulphatase is shown in Fig. 5.

Incubations were carried out at 37° for 2 hr. with androsterone sulphate and dehydro*epi*androsterone sulphate (DHAS) as the substrates; and at 45° for 17 hr. with aetiocholanolone sulphate as substrate. The latter conditions were suggested by Dr I. E. Bush (personal communication). Certain species were also tested for androsterone sulphate activity under these conditions. In addition, if hydrolysis (1–2%) of androsterone sulphate or DHAS took place after 2 hr. the assay was repeated with a 17 hr. incubation period to check whether or not the results of the first assay were significantly different from zero. All three substrates are stable under these conditions between pH 3·0 and 8·0. The effect of increasing the enzyme concentrations from 0·3 to 3·0 mg./ml. on the hydrolysis of DHAS was studied with the powder prepared from *Helix pomatia* crop fluid. Enzymic activity increased linearly up to a concentration of 0·9 mg./ml. and then curved asymptotically. Enzymic activity was assayed on 1·5 mg. of the powder prepared from each test species/ml.

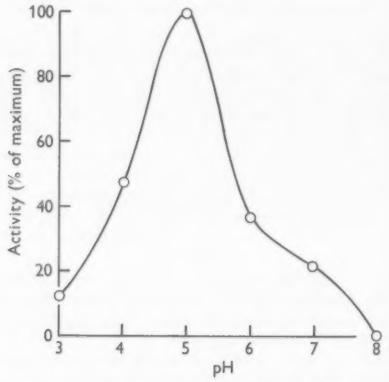

Fig. 5. Effect of pH on steroid-sulphatase activity of *H. pomatia* crop fluid. The volume of the reaction mixture was 2·0 ml., comprising 0·5 ml. of enzyme solution, 1·0 ml. of DHAS solution, and 0·5 ml. of 0·5 M buffers of various pH values. Incubation was for 2 hr. at 37°.

RESULTS

The activities of steroid sulphatase, arylsulphatase and β-glucuronidase from various marine molluscs are summarized in Table 1. *H. pomatia*, the only terrestrial species, has been included for comparison because this animal is commonly used as a source of enzymes for the hydrolysis of steroid conjugates.

The values shown for arylsulphatases and β-glucuronidase were derived from duplicate estimations, and for steroid sulphatases from single estimations, of the acetone-dried powder prepared from each species. The possible influence of factors such as season and sex on enzymic activity was not taken into account. In addition, arylsulphatases and β-glucuronidase were assayed with the pH optima and optimum substrate concentrations found for *P. vulgata*, and no allowance was made for possible variation among the different species. However, the optimum pH found for the hydrolysis of *p*-nitrophenyl sulphate in this study was the same as that reported by Roy (1956*a*) for a much purer enzyme preparation from *P. vulgata* with 2-hydroxy-5-nitrophenyl sulphate as substrate; it also agrees with the value found by Dodgson *et al.* (1953), for homogenates of tissues from *P. vulgata* and *L. littorea* and potassium *p*-acetylphenyl sulphate as substrate. It is therefore possible that there is little variation in pH optima for the hydrolysis of arylsulphates by the enzymes prepared from molluscan tissues. Dodgson *et al.* (1953) reported results for the arylsulphatase and β-glucuronidase present in tissue homogenates prepared from *P. vulgata*, *L. littorea* and *Buccinum undatum*. The results of the β-glucuronidase assays in Table 1 (when converted into units/g. of tissue) are all within the ranges reported by the above workers. Arylsulphatase values cannot be compared because of the different substrates used.

The optimum pH of 4·0–5·0 for the hydrolysis of DHAS by acetone-dried powders prepared from gastropods is in agreement with the pH optimum reported by Savard *et al.* (1954) for *O. punctata* (pH 4·5–5·0), and that reported by Roy (1954) for *P. vulgata* (pH 4·5). Roy (1956*a*), using the steroid sulphatase of *P. vulgata*, has also reported the optimum substrate concentrations for the hydrolysis of DHAS (0·2 mM), *epi*androsterone sulphate (> 0·2 mM) and pregnenolone sulphate (0·06–0·08 mM). As might be expected, the optimum substrate concentration varies with the substrate used. The concentrations of steroid sulphates used in this study (Table 1) were not optimum but were sufficiently high to detect whether or not steroid sulphatases were present.

Attempts were made to increase the rate of hydrolysis of DHAS by the enzyme from *H. pomatia* crop fluid by using possible activators. The influence of two compounds which are known to enhance the activity of β-glucuronidase was investigated. Albumin had no effect, and propylene glycol [which acts as a glucuronic acid acceptor in enhancing β-glucuronidase activity (Fishman & Green, 1956)], depressed the steroid-sulphatase activity by 47%. Barium chloride, which might be expected to increase the rate of hydrolysis of sulphates by removal of sulphate ions, had no effect.

DISCUSSION

The present study has confirmed previous observations on the widespread occurrence of arylsulphatase and β-glucuronidase among marine molluscs. From this survey, however, it appears that steroid sulphatase is confined to one class of the molluscs, the gastropods, and even in this class steroid sulphatase is not invariably found. No correlation could be found between the activity of the arylsulphatases and steroid sulphatases, and neither of these activities could be correlated with that of β-glucuronidase.

Various terms have been used to describe the enzyme present in *P. vulgata* which hydrolyses DHAS. Stitch & Halkerston (1953*a*) describe the enzyme as an 'alkyl sulphatase', whereas Roy (1956*a*) uses the more specific term '3β-steroid sulphatase'. The present study of steroid sulphatases shows that the very high specificity of the enzyme from *P. vulgata* is not found in all gastropods. Three of the species studied were shown to possess enzymes which could hydrolyse aetiocholanolone sulphate as well as DHAS, but an enzyme which would hydrolyse androsterone sulphate was not detected. Species possessing aetiocholanolone sulphate activity (*H. pomatia*, *B. undatum* and *Nassarius reticulatus*) were those which showed the highest specific activity with respect to the hydrolysis of DHAS. This raises the question whether the same enzyme acts on both substrates, DHAS being more readily hydrolysed because of its structural configuration. If this were so, it might be expected that the hydrolysis of androsterone sulphate could only be achieved by an enzyme having very high steroid-sulphatase activity. However, it was found that a powder D (Dodgson & Spencer, 1953), prepared from *P. vulgata*, would not hydrolyse aetiocholanolone sulphate, although possessing an activity three times that of powder A with respect to the hydrolysis of DHAS. Moreover, the highly purified preparation of *P. vulgata* used by Roy (1956*a*) would not hydrolyse aetiocholanolone sulphate. These findings are consistent with the view that two separate enzymes are involved in the hydrolysis of DHAS and aetiocholanolone sulphate, but

Table 1. *Arylsulphatase, β-glucuronidase and steroid-sulphatase activities of various molluscs*

Arylsulphatase activity was determined at a final substrate concentration of 0·012M-2-hydroxy-5-nitrophenyl sulphate (NCS) or 0·03M-*p*-nitrophenyl sulphate (NPS), both at pH 5·5 in 0·5M-acetate buffer. β-Glucuronidase activity was determined at a final substrate concentration of 0·045M-*p*-chlorophenyl glucuronide at pH 4·0 in 0·5M-acetate buffer. Activities of β-glucuronidase and arylsulphatase are expressed in mg. of phenol liberated/g. of powder A/hr. Incubations were for 1 hr. at 37·5°. Steroid-sulphatase activity was determined at a final substrate concentration between 0·065 and 0·08 mM-DHAS, aetiocholanolone sulphate (AeS) and androsterone sulphate at stated pH in 0·125M-buffers, and with the incubation conditions described in the text. Activities are expressed as μg. of steroid liberated/g. of powder A. Androsterone sulphate activity was tested at 37·5° for 2 hr. for all species and at 45° for 17 hr. for species marked *; no activity was detected.

Species	Source of enzyme	Arylsulphatase		β-Glucuronidase	Steroid sulphatase at pH						
		NCS	NPS			3	4	5	6	7	8
Helix pomatia	Digestive gland	760	367	231	AeS	0	1 206	2 261	301	301	0
					DHAS	0	891	1 400	1 273	891	891
	Crop fluid*	2551	823	58	AeS	904	1 507	8 138	2 562	2 261	0
					DHAS	1 018	3 946	8 147	3 055	1 782	0
Patella vulgata	Visceral hump	652	157	156	AeS	0	0	0	0	0	0
					DHAS	0	1 006	2 012	1 868	862	431
Littorina littorea	Whole animal less shell	586	169	69	AeS	0	0	0	0	0	0
					DHAS	509	891	637	382	0	0
*Buccinum undatum**	Digestive gland	228	81	15	AeS	1 055	6 631	12 056	1 808	754	301
					DHAS	431	3 592	2 299	1 581	862	0
*Aplysia punctata**	Digestive gland	287	55	29	AeS	0	0	0	0	0	0
					DHAS	0	0	0	0	0	0
*Nucella lapillus**	Digestive gland	96	39	15	AeS	0	0	0	0	0	0
					DHAS	0	2 465	2 900	1 015	1 015	1 015
*Nassarius reticulatus**	Digestive gland	436	236	76	AeS	2 230	10 409	6 394	595	466	0
					DHAS	5 250	10 650	10 650	2 700	1 200	0
Patina pellucida	Whole animal	26	7	24	AeS	0	0	0	0	0	0
					DHAS	0	450	900	900	750	0
Turritella communis	Whole animal	181	37	0	AeS	0	0	0	0	0	0
					DHAS	0	0	0	0	0	0
Crepidula fornicata	Digestive gland	74	9	8	AeS	—	—	—	—	—	—
					DHAS	0	0	0	0	0	0
Pecten maximus	Digestive gland	941	449	17	AeS	0	0	0	0	0	0
					DHAS	0	0	0	0	0	0
Sepia officinalis	Digestive gland	59	19	10	AeS	—	—	—	—	—	—
					DHAS	0	0	0	0	0	0
Lepidochitona cinerea	Whole animal	41	25	11	AeS	0	0	0	0	0	0
					DHAS	0	0	0	0	0	0

attempts to confirm this by separating the enzymes by starch electrophoresis have been unsuccessful. In this paper the term 'steroid sulphatase' is used to describe any enzyme which will hydrolyse a steroid sulphate. The use of this term is not intended to imply that the physiological role of these enzymes in the Mollusca is to hydrolyse steroid sulphates, as their function is at present obscure.

From the data of Kellie & Wade (1957) it can be calculated that, of the urinary 17-oxo steroid fraction which is not hydrolysed when treated with β-glucuronidase, but which is hydrolysed when the urine is continuously extracted with ether at pH 1 for 72 hr., 70% is dehydro*epi*androsterone, 20% is androsterone and 10% is aetiocholanolone. Presumably this fraction consists of steroids conjugated with sulphuric acid. Further, androsterone sulphate has been isolated from the urine of a patient with an interstitial-cell tumour of the testis (Venning, Hoffman & Browne, 1942). Thus enzymic hydrolysis of all the steroid sulphates present in human urine cannot be achieved with any of the enzyme sources studied in this work. In particular, androsterone sulphate is unaffected, and this possibly means that 3α-sulphates of other 3α:5α compounds are not hydrolysed either. The use of enzyme preparations from *P. vulgata* in the quantitative determination of urinary 17-oxo steroids (see Stitch & Halkerston, 1956) cannot be recommended, since these will not hydrolyse androsterone sulphate or aetiocholanolone sulphate. Moreover, it is unlikely that sulphatases of fungal and bacterial origin would hydrolyse the neutral 17-oxo steroids because mylase P and Taka-diastase (Cohen & Bates, 1949; Stitch & Halkerston, 1953*b*) and microbiological sulphatases (Buehler, Katzman & Doisy, 1950) are ineffective. The steroid sulphatases prepared from mammalian sources, e.g. rat and ox liver, have the same specificity requirements as those of the enzyme from *P. vulgata*, but exhibit a much lower activity with respect to the hydrolysis of DHAS (Gibian & Bratfisch, 1956; Roy, 1957).

The most convenient marine sources of enzymes for use in analytical work with conjugated steroids seem to be *B. undatum* and *N. reticulatus*, for preparations made from these animals hydrolyse both aetiocholanolone sulphate and DHAS and possess considerable β-glucuronidase and arylsulphatase activity. However, the sulphatases have no measurable influence on the hydrolysis of androsterone sulphate and a suitable source of material capable of effecting this process has yet to be found. Preliminary results on species from invertebrate phyla other than the Mollusca (Corner, Leon & Bulbrook, 1960) indicate that steroid sulphatase is absent.

SUMMARY

1. Acetone-dried powders prepared from thirteen species of molluscs have been tested for steroid-sulphatase activity with the sulphates of dehydro*epi*androsterone, androsterone and aetiocholanolone as substrates.

2. Three species (*Helix pomatia*, *Buccinum undatum* and *Nassarius reticulatus*) were found to possess activity towards both dehydro*epi*androsterone sulphate and aetiocholanolone sulphate, whereas in four species (*Patella vulgata*, *Littorina littorea*, *Patina pellucida* and *Nucella lapillus*) activity towards only dehydro*epi*androsterone sulphate was found. Steroid-sulphatase activity was not detected in six species.

3. No enzyme capable of hydrolysing androsterone sulphate was found in any of the animals tested; therefore complete enzymic hydrolysis of urinary steroid conjugates cannot be achieved with enzyme preparations from these molluscs.

4. β-Glucuronidase and arylsulphatase activity are widely distributed in the Mollusca, but the activities of these enzymes are not related to the activity of steroid sulphatase.

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Canavanine in the Leguminosae

By E. A. BELL
Department of Biochemistry, King's College, London, W.C. 2

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Canavanine (α-amino-δ-guanidoxybutyric acid) was originally discovered in three closely related species of *Canavalia* (Kitagawa & Tomiyama, 1929; Kitagawa, 1937; Damodaran & Narayanan, 1939). Subsequently it was identified in the seeds of 17 other leguminous plants and isolated from three of them (Fearon & Bell, 1955; Bell, 1958). Evidence is now given for the presence of canavanine in the seeds of other leguminous plants.

Bell (1958) suggested that canavanine stored in leguminous seeds provides a readily available supply of nitrogen for the developing embryo. It is now shown that the concentration of canavanine in the seeds of *Medicago sativa* does in fact fall rapidly during germination.

The apparent restriction of canavanine to the Leguminosae (Fearon & Bell, 1955) and its occurrence in 31 plants of this group suggested that its biosynthesis might be directly or indirectly associated with the presence of nitrogen-fixing bacteria in the root nodules of these plants. Canavanine has therefore been tested for in both the cells and growth media of bacteria capable of fixing nitrogen whilst living in the free state and also of bacteria known to fix nitrogen while living in symbiotic association with leguminous plants. Plants of *M. sativa* and *Trifolium pratense* grown in the presence and absence of their respective rhizobia, and the seeds of certain non-leguminous plants reported to fix nitrogen, have been tested for canavanine.

EXPERIMENTAL

Pentacyanoammonioferrate reagent and phosphate buffer (pH 7). These were prepared as described by Bell (1958).

Qualitative analysis of seeds

Preparation of seed extracts. Finely ground seed (0·1–0·3 g.) was stirred with 1 ml. of 0·1 N-HCl at room temperature and kept for 18 hr. The suspension was then neutralized with 1 ml. of 0·1 N-NaOH and the supernatant was used for paper chromatography and ionophoresis.

Chromatography. The solvents used were phenol–water (4:1, w/v), butanol–pyridine–acetic acid–water (4:1:1:2, by vol.) and solvent F of Reio (1958) [methyl ethyl ketone–acetone–water–formic acid (80:4:12:2, by vol.)]. Chromatograms were run on Whatman no. 1 paper for 24 hr. in the first two solvents and for 5 hr. in the third. The chromatograms run in the second solvent were sprayed with phosphate buffer and redried in air to remove the last traces of pyridine (Bell, 1958). All chromatograms were sprayed with pentacyanoammonioferrate (PCAF) reagent, which gives a characteristic magenta spot with canavanine.

Ionophoresis. Ionophoresis was carried out at pH 7 as described by Bell (1958), and the dried papers were sprayed with PCAF reagent.

PCAF reaction negative. Extracts of the seeds of the following plants gave no colour with PCAF.

Leguminosae: *Baptisia australis, Cercis siliquastrum, Clitoria ternatea, Cytisus albus, C. praecox, C. scoparius, C. sessilifolius, Dolichos lablab, Genista hispanica. G. sagittalis, G. tinctoria, Glycine soja, Laburnum anagyroides, Lespedeza buergeri, Lupinus arboreus, L. littoralis, L. nootkatensis, L. polyphyllus, L. subcarnosus, Melilotus alba, Mimosa pudica, Onobrychis caput-galli, Petteria ramentacea, Spartium junceum, Tetragonolobus purpureus, Vicia angustifolia, V. lutea, V. sativa, Vigna cylindrica.*

Coriariaceae: *Coriaria terminalis, C. thymaefolia.*

Elaeagnaceae: *Elaeagnus angustifolia, E. pungens reflexa, E. umbellata, Hippophae rhamnoides.*

PCAF reaction positive. A compound giving a magenta reaction with PCAF at pH 7 and occupying the same position as authentic canavanine on the chromatography and ionophoresis papers was found in the seeds of the following Leguminosae: *Astragalus alopecuroides, A. cicer, Caragana arborescens, Colutea media, Lotus uliginosus, Medicago falcata, M. lupulina, Ononis rotundifola, Securigera securidaca, Trifolium pratense, Vicia hirsuta.*

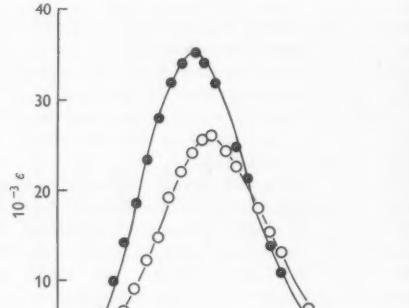

Fig. 1. Absorption spectra of canavanine–PCAF (●) and deaminocanavanine–PCAF (○). Solutions were prepared as described in the text.

Absorption spectra

Canavanine–PCAF. To 0·25 mg. of canavanine in 10·5 ml. of buffer solution was added 0·1 ml. of PCAF reagent. The solution was kept for 40 min., after which time the colour had fully developed. The absorption spectrum was determined in a Unicam SP. 500 spectrophotometer with a mixture of 10·5 ml. of buffer and 0·1 ml. of PCAF reagent as 'blank'. The curve obtained (Fig. 1) showed a single peak with a maximum at 522 mμ.

Deaminocanavanine–PCAF. To 0·32 mg. of deaminocanavanine (hexahydro-3-imino-1-oxa-2:4-diazepine-5-carboxylic acid) in 10·5 ml. of buffer solution was added 0·1 ml. of PCAF reagent. The solution was kept in the dark for 2 hr. The deaminocanavanine colour developed more slowly than the canavanine colour and was less stable in daylight. This compound showed a single absorption maximum (Fig. 1) at 536 mμ. Deaminocanavanine is not known to occur naturally in the Leguminosae but is formed by the decomposition of canavanine in aqueous solution even at 30°.

Estimation of canavanine

For the determination of canavanine in the individual seeds of *M. sativa* the sensitivity of the colorimetric method of Fearon & Bell (1955) was improved by using a spectrophotometer and restricting measurements to the absorption maximum. By these means a concentration–extinction curve was prepared with canavanine standards of approximately one-tenth of the original concentration. The smaller cell size of the instrument also contributed to an overall increase of some 30-fold in sensitivity. A linear curve passing through the origin was obtained up to a concentration of 0·14 m-mole/l., at which the extinction in a 1 cm. light path was 0·52.

Disappearance of canavanine from seeds of Medicago sativa *during germination*

Seeds of the same batch were used for all experiments.

Canavanine content of seeds. Seeds (20, weight range 2·0–3·2 mg.) of *M. sativa* were weighed separately, ground and triturated with 3 ml. of buffer solution. To each suspension was added 0·1 ml. of PCAF reagent; after 2 hr. the suspensions were filtered and the extinction of each filtrate was determined at 522 mμ. The canavanine content was between 0·5 and 1·4 % of the weight of the seeds examined. Bell (1958) found 1·46 % for a batch of seed which had been ground, mixed and dried at 110° for 20 hr. before analysis. Measurements made after adding known weights of canavanine confirmed that 0·001 mg. of the amino acid could be determined in the seed extracts.

Germination of seeds on sand. Seeds of *M. sativa*, of the same weight range as those analysed, were surface-sterilized with concentrated sulphuric acid and, after washing 10 times with sterile water, were allowed to germinate in individual tubes. One group of seeds was supported on 'slopes' of fine sand covered at the lower end with water. A second group was grown on sand watered with a solution of mixed salts containing (%): $CaHPO_4$, 0·1; K_2HPO_4, 0·02; NaCl, 0·02; $MgSO_4,7H_2O$, 0·02; $FeCl_3$, 0·01; and (p.p.m.) $Na_2MoO_4,2H_2O$, 2·5; $CuSO_4,5H_2O$, 3·9; $MnSO_4$, $4H_2O$, 9·3; $ZnSO_4,7H_2O$, 4·4; H_3PO_3, 5·6. Ammonium sulphate equivalent to 2 mg. of N/seed was also added to each tube of this group. All tubes were sterilized in the autoclave before the seeds were introduced. After developing on the sand 'slopes' for 7 days, 40 seedlings which had been supplied with the mixed-salts solution and $(NH_4)_2SO_4$ were removed from their tubes. A further 40 seedlings were removed after 14 days' growth. The sand in each tube was filtered and washed free of adhering liquid with buffer solution. The combined filtrate and washings were made up to 3 ml. To each tube was added 0·1 ml. of PCAF reagent and 40 min. was allowed for colour development. No canavanine was detected in any of the 80 tubes.

Each of the seedlings was crushed and triturated with 3 ml. of buffer solutions; 0·1 ml. of PCAF reagent was added to each suspension and 40 min. allowed for colour development. Of the seedlings grown for 7 days, six of those supplied with water and seven of those supplied with salts solution gave a positive reaction for canavanine. Of those grown for 14 days, four supplied with water and two supplied with salts solution gave a positive reaction. No colour developed in the remaining tubes on further keeping.

The addition of canavanine (one ground ungerminated seed) to each of the seedling extracts which gave a negative reaction and to each of the tubes of media produced a positive result. The sensitivity of the PCAF test in the presence of the crushed seedlings was determined by adding canavanine to the extracts. An amount of 0·004 mg. of canavanine was readily detectable.

Growth of seedlings on an agar medium. Sterile seeds of *M. sativa* and *T. pratense* were sown individually in test tubes on 'slopes' containing 1·2 % of agar (Difco Bacto) and inorganic salts in the same proportions as in the mixed-salts solution described. The medium was adjusted to pH 6·6–6·7 with 0·1 N-NaOH. The seeds were divided into four groups each containing 20 of each species. To one group was added $NaNO_3$ (2 mg. of N/tube) and to a second group was added an equivalent weight of $(NH_4)_2SO_4$. The seeds of *M. sativa* in the third group were innoculated with *Rhizobium meliloti* (Rothamsted AH_1) and those of *T. pratense* with *Rhizobium trifolii* (Rothamsted ClF). The remaining seeds were retained as controls. Sterile water and a diluted mixed-salts solution were added to the plants as required during growth. Half the plants were analysed after 6 weeks' and the remainder after 10 weeks' growth. The whole plants were extracted with 3 ml. of buffer solution and treated with 1 ml. of PCAF reagent. No canavanine was detected in any group after 6 weeks' growth. Positive results were obtained only with 10-week plants to which $(NH_4)_2SO_4$ or $NaNO_3$ had been added. These results are in agreement with the finding that both nodulated and non-nodulated plants of *M. sativa* grown for 17 weeks with added NH_4NO_3 under field conditions gave a positive test for canavanine.

Qualitative analysis of bacterial extracts and media

Azotobacter vinelandii and *A. chroococcum* were grown in a liquid medium containing (%); mannitol, 2·0; K_2HPO_4, 0·3; $CaCO_3$, 0·1; NaCl, 0·1; $FeCl_3$, 0·001; $MgSO_4,7H_2O$, 0·001; and (p.p.m.) $Na_2MoO_4,2H_2O$, 1·0. The pH was adjusted to 7·2 with 0·1 N-NaOH. *Rhizobium meliloti* was grown in a medium containing (%): sucrose, 0·5; yeast extract (Yeastrel), 0·5; K_2HPO_4, 0·5; $MgSO_4,7H_2O$, 0·1; $FeCl_3$, 0·001; Na_2MoO_4, $2H_2O$, 0·0001; the pH was adjusted to 7·0 with 0·1 N-NaOH. After time had been allowed for the

colonies to establish themselves, 7, 10 and 21 days respectively, the bacteria were centrifuged and the culture medium was decanted. Part of each sediment of bacterial cells (0·5–1·0 ml.) was ground with fine sand and stirred with 2–3 ml. of 0·1 N-HCl. After 1 hr. the supernatant liquid and the decanted culture medium were examined by paper chromatography. No canavanine was detected in either the cell extracts or the culture media.

SUMMARY

1. Canavanine has been detected in the seeds of 11 species of leguminous plants.

2. The sensitivity of the method for the colorimetric determination of canavanine has been increased by using a spectrophotometer at 522 mμ.

3. Canavanine stored in seeds of *Medicago sativa* is metabolized by the seedlings during germination and growth.

4. The biosynthesis of canavanine by *Medicago sativa* and *Trifolium pratense* is not dependent upon the presence of nitrogen-fixing bacteria in the root nodules of the plants.

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Spectral-Absorption Coefficients of some Porphyrins in the Soret-Band Region

By C. RIMINGTON
Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

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All porphyrins exhibit intense fluorescence when illuminated by light of appropriate wavelength and this property has been much used in the past for their quantitative determination. The intensity of the fluorescence varies with pH (Fink & Hoerburger, 1933, 1935; Rimington, 1943; Jope & O'Brien, 1945), however, and is also much affected by impurities and even by the presence of inorganic ions. Notwithstanding the use of a reference standard for each determination, errors may be large and unpredictable.

With the advent of modern photoelectric spectrophotometers, it has become possible to exploit for quantitative measurement another characteristic physical property of porphyrins, namely their intense spectral absorption at about 400 mμ. This band, known as the Soret band, is intense and narrow and is most well developed in acid solution; its position and magnitude differ for each porphyrin. Both parameters are influenced by pH (cf. Jope & O'Brien, 1945) but conditions for measurement are easily standardized and non-absorbing impurities are without effect. For measurement in the presence of substances which absorb light, a correction formula has been devised by Rimington & Sveinsson (1950). This is generally applicable so long as the absorption of the impurities remains approximately linear over a narrow spectral range on either side of the Soret maximum of the porphyrin in question.

The porphyrins in acid solutions obey Beer's law up to an extinction (optical density) of about 1·0, and spectrophotometry is thus the method of choice for their determination. It is necessary, however, to know the molecular extinction coefficients (ϵ) of the pure materials under the conditions which are to be applied for their measurement. Unfortunately the preparation of porphyrins or their methyl esters in the pure state is difficult; melting points are unsatisfactory criteria of purity and it is thus not surprising that widely differing values for ϵ or $E^{1\%}_{1\,cm.}$ are to be found in the literature. The situation has been reviewed briefly by With (1955). The purpose of the present paper is to present the values found by examination of a number of carefully purified porphyrins and so to provide data for accurate spectrophotometry. Measurements have been made upon porphin and aætioporphyrin I for comparison with the naturally occurring porphyrins.

MATERIALS AND METHODS

Measurements of extinction were made in a Unicam model SP. 500 photoelectric spectrophotometer. Purification of the porphyrin methyl esters was effected by repeated crystallization. A weighed quantity was then dissolved in freshly washed and dried chloroform or other solvent as specified in Table 1 and the Soret band was measured. For examination of the free porphyrins, another weighed quantity was left in contact with 7N-HCl for 48 hr., after which the solution was diluted to appropriate concentration of acid and porphyrin for spectrophotometric measurement. With aetioporphyrin I a measured quantity of a concentrated solution in dioxan was diluted appropriately with HCl so that the final acid concentration was 1·37N. N.P.L. grade A glassware was used throughout.

Porphin. Synthetic material (Krol, 1959; Rimington, Mason & Kennard, 1958) was used.

Aetioporphyrin I. This was synthetic material from Hans Fischer's collection.

Uroporphyrin III. This was synthetic octamethyl ester supplied by Dr S. F. MacDonald. Natural material was prepared from turacin (Nicholas & Rimington, 1951).

Coproporphyrin III. This was prepared from residues obtained in production of toxoid from cultures of *Corynebacterium diphtheriae* (Gray & Holt, 1948).

Deuteroporphyrin IX. This was prepared synthetically from haemin (Fischer & Orth, 1937).

Protoporphyrin IX. This was prepared by the method of Grinstein (1947) from haemin.

Phylloerythrin. This was isolated from sheep's faeces (Rimington & Quin, 1934).

RESULTS

Calculated values for ϵ and $E^{1\%}_{1\,cm.}$ obtained in this investigation are presented in Tables 1 and 2, together with values from the literature.

DISCUSSION

There is reason to believe that the molecular extinction coefficients of porphyrin isomers do not differ significantly from one another. Thus uroporphyrin esters I and III afford substantially the same figure (Rimington & Sveinsson, 1950), and the bands of coproporphyrin esters I and II in the visible region are practically identical (Stern & Wenderlein, 1934), as are the bands of coproporphyrins I, II, III and IV in 3N-hydrochloric acid (Stern & Wenderlein, 1934). Jope & O'Brien (1945) found the same molecular extinction coefficient for coproporphyrins I and III in 0·1N-hydrochloric acid.

Examination of Tables 1 and 2 reveals generally good agreement between the figures here reported and those to be found in the literature which are based upon direct measurement. The somewhat higher figure found for protoporphyrin in acid solution by Grinstein & Wintrobe (1948) is probably preferable to that given by the specimen used in this work. A real difficulty arises, however, when the values for coproporphyrin are considered. Jope & O'Brien (1945) in their study of the I and III isomers used photographic apparatus and reported ϵ values of $5{\cdot}31 \times 10^5$ and $5{\cdot}30 \times 10^5$ respectively for the Soret band in 0·1N-hydrochloric acid. This figure has never been attained by any subsequent investigator; in fact all careful measurements lie around $4{\cdot}8$–$4{\cdot}9 \times 10^5$. It seems probable therefore that Jope & O'Brien's figure is erroneous. It is important to have an agreed extinction value for use in calculating coproporphyrin concentrations and the adoption of $\epsilon\ 4{\cdot}89 \times 10^5$ for the Soret band in 0·1N-hydrochloric acid as solvent is recommended as a result of this study. This figure is almost identical with that found by Zondag & Van Kampen (1956).

Absorption of energy manifested in the Soret band is attributable to a single transition affecting the resonating system of the porphyrin ring structure as a whole (Rabinowitch, 1944). Features such as the vinyl groups of protoporphyrin and the isocyclic ring of phylloerythrin would be expected therefore to influence considerably the intensity of this absorption band. The effect of alkyl or carboxyalkyl substituents in the β positions may be elucidated by consideration of the data in Tables 1 and 2. Table 1 records absorption of porphin, aetioporphyrin I and porphyrin esters in neutral solvents and Table 2 gives data for the free porphyrins in acid, arrangement being in order of decreasing ϵ values. The individuals are placed in the same order in Table 1.

Effect of alkyl substitution. Porphin, with all eight β positions unsubstituted, has the highest ϵ of all the porphyrins examined. Substitution with symmetrically arranged electron-repelling methyl and ethyl groups, as in aetioporphyrin I, leads to a profound depression of the molar extinction coefficient whereas substitution with carboxyalkyl groups has less effect. The ϵ of uroporphyrin octamethyl ester lies approximately midway between the values for porphin and aetioporphyrin I; the values for coproporphyrin tetramethyl ester and the dicarboxylic porphyrin esters are considerably lower and approach that for aetioporphyrin I.

Effect of acid medium and of carboxyl groups. All molar extinctions are enhanced when the free porphyrin is dissolved in acid. Thus ϵ of porphin rises from $2{\cdot}64 \times 10^5$ to $3{\cdot}48 \times 10^5$ (1·32 times) but the effect is even greater with the alkyl- and

carboxyalkyl-substituted porphyrins. In these ϵ increases from 2·4 to 2·7 times, except in the special cases of phylloerythrin (1·42 times) and protoporphyrin (1·54 times). The enhanced intensity of absorption in an acid medium is clearly due to the symmetry introduced by protonation of the pyrrolic nitrogen atoms.

Values of the constant for use in the correction formula

In 1950, Rimington & Sveinsson (1950) deduced an expression

$$P_{max.} = \frac{2\,D_{max.} - (D_{430} + D_{380})}{k}$$

Table 1. *Extinction coefficients of porphyrin methyl esters, porphin and aetioporphyrin I*

Solvent: chloroform, unless otherwise indicated.

Material	Soret max. (mμ)	$10^{-3}\,E^{1\%}_{1\,cm.}$	$10^{-5}\,\epsilon$	$10^{-5}\,\epsilon$ values from literature
Uroporphyrin III octamethyl ester (syn.)	405–6	2·30	2·17	—
Uroporphyrin I octamethyl ester (nat.)	406	—	—	2·14,* 1·95†
Coproporphyrin III tetramethyl ester	399·5	2·53	1·80	1·80‡
Aetioporphyrin I‖	399·5	3·35	1·60	—
Deuteroporphyrin dimethyl ester	399	3·25	1·75	—
Haematoporphyrin dimethyl ester	402	—	—	1·75§
Porphin¶	396·5	8·52	2·64	—
Phylloerythrin methyl ester	415	3·57	1·95	—
Protoporphyrin dimethyl ester	407·5	2·89	1·71	—

* S. Granick (personal communication) on uroporphyrin I octamethyl ester. One specimen supplied by Rimington, one by With.
† Rimington & Sveinsson (1950).
‡ Calculated from data of Brugsch & Kubowitz (1953) for coproporphyrin I tetramethyl ester in dioxan.
§ Granick, Bogorad & Jaffe (1953) for the diester in pyridine.
‖ In dioxan.
¶ In benzene.

Table 2. *Extinction coefficients of porphyrins in acid solution*

Material	Concn. of HCl (N)	Soret max. (mμ)	$10^{-3}\,E^{1\%}_{1\,cm.}$	$10^{-5}\,\epsilon$	$10^{-5}\,\epsilon$ values from literature
Uroporphyrin III (syn.)	0·5	405·5	6·36	5·28	—
Uroporphyrin I (nat.)	0·5	—	—	—	5·41*
Coproporphyrin III	0·1	399·5	7·47	4·89	4·78†
	0·1	—	—	—	4·85‡
	0·1	—	—	—	5·30§
Deuteroporphyrin	0·1	398	8·48	4·33	—
Haematoporphyrin	0·5	401	—	—	4·23‖
Porphin	1·37	391	11·23	3·48	—
Phylloerythrin	3·0	421	5·08	2·78	—
Protoporphyrin	1·37	408	4·67	2·62	2·75¶

* Rimington & Sveinsson (1950).
† Calculated from data of Brugsch & Kubowitz (1953) for coproporphyrin I.
‡ Zondag & Van Kampen (1956). A second determination gave $4{\cdot}87 \times 10^{-5}$. (Personal communication from Dr Van Kampen).
§ Jope & O'Brien (1945).
‖ Legrand (1955). Solvent 0·5N-H_2SO_4.
¶ Grinstein & Wintrobe (1948).

Table 3. *Recommended data for determination of porphyrins by measurement of the Soret band*

Data are included for correction for absorbing impurities according to Rimington & Sveinsson (1950).

Porphyrin	Concn. of HCl (N)	$10^{-5}\,\epsilon$	$10^{-3}\,E^{1\%}_{1\,cm.}$	Extremes for measurement of D in formula (mμ)	k
Uroporphyrin	0·50	5·41	6·52	380, 430	1·844
Coproporphyrin	0·10	4·89	7·47	380, 430	1·835
Deuteroporphyrin	0·10	4·33	8·48	375, 425	1·868
Protoporphyrin	1·37	2·75	4·89	380, 430	1·668
Phylloerythrin	3·00	2·78	5·08	395, 445	1·753

which could be used to eliminate the effect of impurities in measurements made on porphyrins in acid solutions containing absorbing impurities. The expression is generally applicable provided that the increase or decrease of absorption due to the impurities remains approximately linear over the range 380–430 mμ. D_{380}, D_{430} and $D_{max.}$ are the optical densities observed at the wavelengths 380, 430 mμ and the peak of the Soret band respectively; $P_{max.}$ is the Soret band absorption actually due to the porphyrin, and k is a factor empirically determined from measurements upon the pure porphyrin (Rimington & Sveinsson, 1950; Zondag & Van Kampen, 1956; With, 1955). This expression was originally worked out for uroporphyrin, the Soret maximum of which in 0·5N-hydrochloric acid lies at 405 mμ and the wavelengths 430 and 380 mμ were adopted as being equidistant from the maximum and near the foot of the band on either side of the peak. For absolute precision, the lateral values should always be equidistant from the peak, thus for example at 425 and 375 mμ for correction of coproporphyrin solutions (Soret maximum approx. 400 mμ), but in practice the error introduced by using 430 and 380 mμ for all the commoner porphyrins, except deuteroporphyrin, is negligible and a standard procedure is much more convenient. In phylloerythrin, the Soret maximum is too far removed from 405 mμ and here also a new expression has to be applied (see Table 3). In all cases, the value of k must be empirically determined and there has been some discussion about the most acceptable values (see With, 1955). In Table 3 the recommended data are summarized for those porphyrins for which they are available.

SUMMARY

1. The spectral absorption in the Soret region of some common porphyrins has been examined.

2. Values are reported for ϵ at the Soret band, of the esters in a neutral solvent and of the porphyrins dissolved in acid of suitable concentration.

3. Recommended values are tabulated for use with the formula of Rimington & Sveinsson (1950), which permits correction for light-absorbing contaminants of porphyrins in impure solutions.

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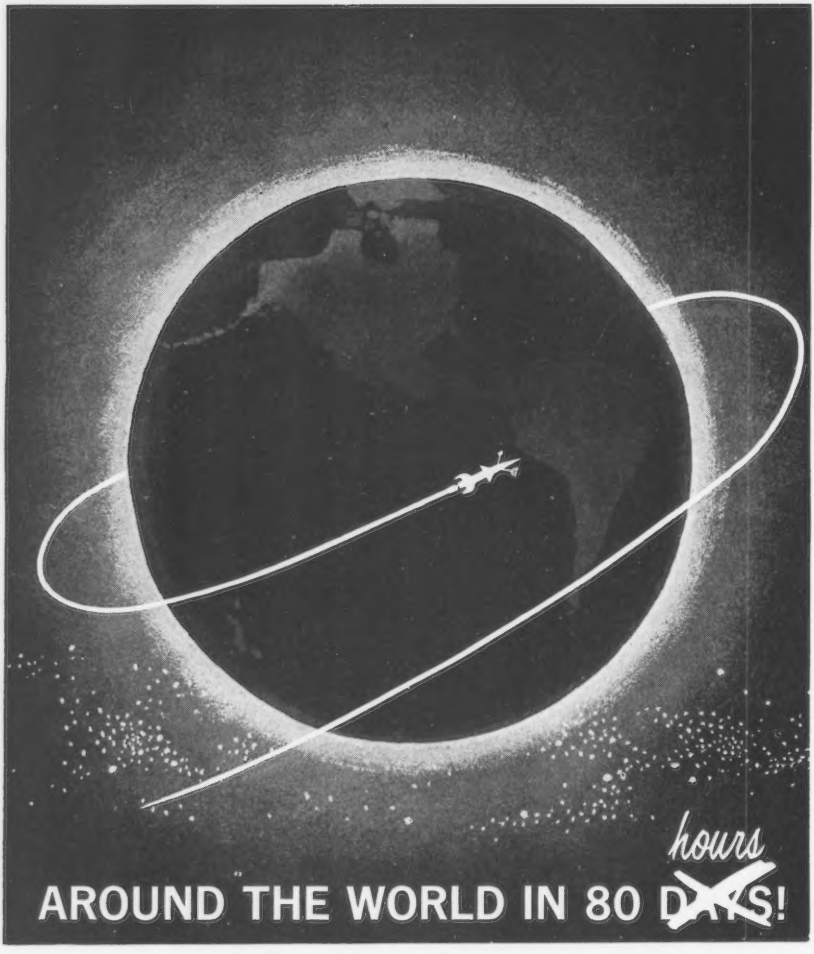

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EXAMPLE

Identification of Intermediate Substrate Free-Radicals formed during Peroxidatic Oxidations

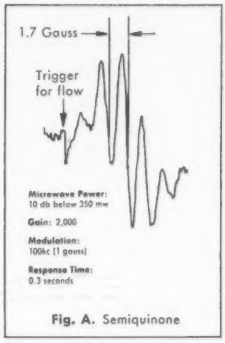

Fig. A. Semiquinone

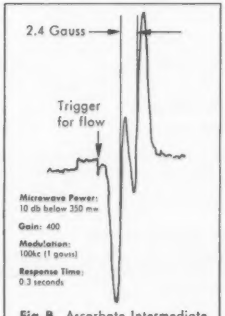

Fig. B. Ascorbate Intermediate

A considerable amount of work utilizing the technique of electron paramagnetic resonance (EPR) to detect free-radical production in typical enzyme-substrate oxidation-reduction reactions has been reported. For the most part the EPR signals that have been observed in the system have been associated with the enzyme itself, or with a change in valance state (i. e., to a paramagnetic state) of the metal in the metalloflavoproteins. In no case has evidence been presented to associate the free radical signal with the substrate undergoing oxidation or reduction. The EPR technique is valuable not only for detecting free radicals but can also be used to identify the type of free radicals in a system and provides a direct measure of their concentration, since the total intensity of the spectral lines is directly proportional to the concentration of free radical intermediates present. With the improved sensitivity and larger sample volumes obtainable, it is now possible to re-investigate free radical production in these biological systems.

In the research described (see illustrations), EPR has been used to detect, identify, and follow the kinetics of free radical formation and decay in the oxidation of ascorbic acid by an enzyme in H_2O_2 solution. These observations were made with a Varian 100 kc EPR Spectrometer, utilizing a flat sample cuvette attached to a flow system for kinetic measurements. The enzyme was recrystallized Japanese turnip peroxidase.*

Figure A illustrates the free-radical spectrum obtained from a mixture of enzyme (8×10^{-8}M) and a solution of hydroquinone (10^{-2}M), H_2O_2 (10^{-2}M) and acetate buffer (pH 4.8). The measured concentration of free radicals from this enzyme reaction was 1.3×10^{-6}M in the steady state.

Figure B shows the intermediate formed during the peroxidatic oxidation of ascorbic acid in a steady state reaction. The concentration of free radicals resulting from the reaction of ascorbic acid (10^{-2}M), H_2O_2 (10^{-2}M), and peroxidase (1.6×10^{-7}M) at pH = 4.8, was 7.2×10^{-6}M.

There is no doubt that the free radicals generated during the peroxidatic oxidations of hydroquinone and ascorbic acid are derived from the substrates.** The enzymic generation of free radicals from substrates, which have been observed in this investigation by electron paramagnetic resonance spectroscopy, suggests that aerobic life may have an inherent genetic instability due to mutations which such free radicals could produce. This interesting possibility seems to merit further investigation.

** Samples courtesy of I. Yamazaki and H. S. Mason, Department of Biochemistry, University of Oregon Medical School, Portland, Oregon, U. S. A.*

*** See: Yamazaki, Mason and Piette; Biochem. and Biophys. Comm; Vol. 1, No. 6, pp. 336-337.*

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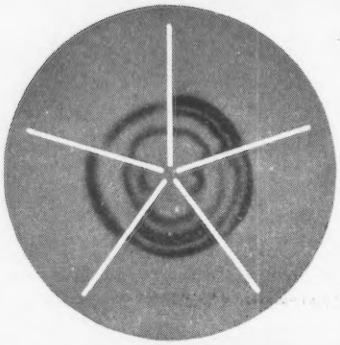

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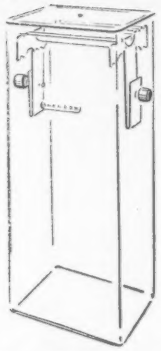

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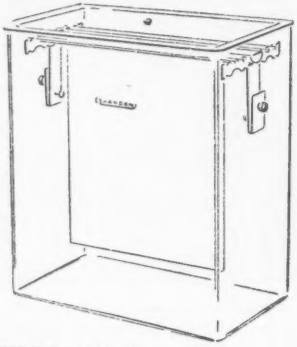

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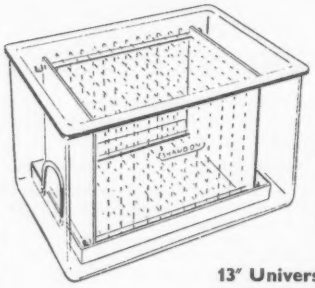

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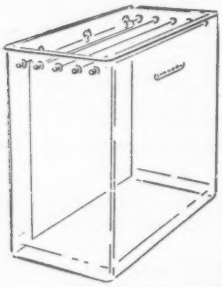

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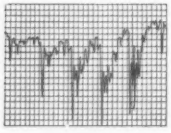

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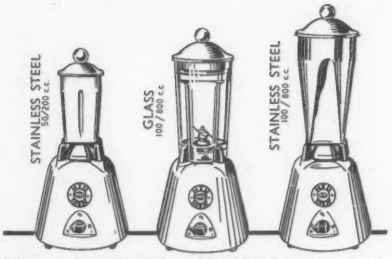

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **391st Meeting** *of the Society was held in The Royal College of Surgeons, London, W.C. 2, on Saturday,* 13 *February* 1960, *when the following papers were read:*

SYMPOSIUM ON THE BIOCHEMISTRY OF MUCOPOLYSACCHARIDES OF CONNECTIVE TISSUE

Chondroitin Sulphates and Sulphated Polysaccharides of Connective Tissue. By HELEN MUIR. (*Medical Unit, St Mary's Hospital, London, W.* 2)

Sulphated polysaccharides are widely distributed in ground substance and may be an important homeostatic constituent of extracellular space, as well as providing connective tissue with characteristic physical properties. The chondroitin sulphates are made up of disaccharide units of uronic acid and galactosamine. They are combined with non-collagenous protein in a manner which is only now beginning to be understood, and which does not appear to depend on electrostatic bonds, although these must contribute to some extent to the stability of the tissue. Chondroitin sulphates cannot normally be brought into solution until the protein–carbohydrate bond is destroyed or until the protein itself is digested with proteolytic enzymes. However, after drastic mechanical disruption of cartilage most of the chondroitin sulphate becomes soluble and is all combined with non-collagenous protein. Extensive physico-chemical studies on a similar material, prepared with less drastic mechanical treatment, have shown that this protein–chondroitin sulphate complex is polydisperse, has a large molecular weight and high viscosity and in solution, the molecules behave as rods. The protein–carbohydrate bond is irreversibly destroyed at pH 12. In a complex comprising a more readily extracted minor component of cartilage, the viscosity is greatly reduced when the protein–carbohydrate bond is broken and when the relatively small protein moiety is digested with enzymes. Proteolysis leaves a peptide fragment still attached to the polysaccharide moiety, and this fragment is similarly labile to alkali. The implications of these findings will be discussed.

Meyer has distinguished three chondroitin sulphates which differ in their optical rotation, susceptibility to hyaluronidase and solubility. Evidence for their distinct identity has recently been found. They are all β-glycosides with negative optical rotations. Chondroitin sulphate *A* differs from *C* in the position of the sulphate group on the galactosamine residue, while *B* contains iduronic acid instead of glucuronic acid. The proportions of these chondroitin sulphates vary in different tissues and there is evidence that they also vary with age.

The other sulphated polysaccharides are less well defined. Keratosulphate, which is found in intervertebral disks, differs from chondroitin sulphate in containing galactose but has no uronic acid. Heparin monosulphate, which occurs in some types of connective tissue, is related to heparin in being probably an α-glycoside, with disaccharide units of glucuronic acid and glucosamine. Unlike heparin it contains one and not about three sulphate groups and the amino group is acetylated.

Unlike the collagen of connective tissue, the sulphated polysaccharides are in an active metabolic state which can be influenced by hormones, while in some experimental and pathological conditions the proportions of these polysaccharides can be greatly altered.

The Histological Demonstration of Mucopolysaccharides. By R. C. CURRAN. (*Department of Pathology, St Thomas's Hospital, London, S.E.* 1)

During the last decade the interest shown by chemists in the structure of connective tissues has been great, mainly as a result of the concept of the 'collagen' diseases. The information which they have gained concerning the fibrillary component of connective tissue has resulted in a more enlightened interpretation of the histological appearances of normal and pathological tissues. However, with regard to the mucopolysaccharides, which form a major part of the amorphous 'ground-substance' of connective tissue, the position is less satisfactory; the gap between the biochemist and the

morphologist is still very great. The newer staining and histochemical methods for mucopolysaccharides devised during the last few years are, however, helping to narrow this gap. With their aid the distribution of mucopolysaccharides in tissue sections can be determined, albeit somewhat crudely; the combination of radioactive sulphate and radioautography has proved particularly useful.

The review is concerned with the various techniques available and is illustrated with examples drawn from several pathological states in man.

The Analysis of Mucopolysaccharides. By S. GARDELL. (*Kemiska Laboratoriet, Serafimerlasarettet, Stockholm, Sweden*)

When mucopolysaccharides are isolated from the connective tissue a mixture of related substances is obtained. The first step in the analysis of the mucopolysaccharides is therefore the separation of this mixture into its components.

Fractional precipitation of the polysaccharides with organic solvents from aqueous solutions containing different metal ions has been the most commonly used method. These methods, however, do not always give clear-cut separations. They are also difficult to use when only small amounts of material are available. Some recent improvements in this technique have been described.

Electrophoretic methods are useful in cases where a distinct difference in electrophoretic mobility exists between the mucopolysaccharides, e.g. between hyaluronic acid and heparin, and between hyaluronic acid and the chondroitin sulphuric acids.

The use of partition methods is restricted because of lack of suitable solvent systems. In some cases this difficulty has been overcome by the use of 'carriers' such as fatty amines. However, both paper-chromatographic methods and column-chromatographic methods have been used.

Quaternary ammonium salts containing a long aliphatic chain give complexes with acidic polysaccharides. These complexes are insoluble in water but are soluble in salt solutions. Moreover, different polysaccharides require different concentrations of salt to have their complexes with the quaternary ammonium compound dissolved.

These principles for the fractionation of mucopolysaccharides have been used in recent work, and chromatography of polysaccharides on modified cellulose ion-exchangers is also a recent innovation.

The next step in the analysis of the mucopolysaccharides is the determination of the monosaccharide components. Compared with other polysaccharides the glycosidic bonds in mucopolysaccharides are very resistant to acid hydrolysis. Generally hydrolysis with 2–6N-hydrochloric acid for 8–20 hr. is needed to reach a maximum hydrolysis.

The amino sugars are not destroyed by this treatment. They can be separated, identified and quantitatively determined from the hydrolysis mixture by ion-exchange chromatography. Paper chromatography of the amino sugars as such, or particularly of the pentoses formed after oxidation with ninhydrin, is a very important technique for identification of the amino sugars.

Two types of uronic acid have been found in mucopolysaccharides, glucuronic acid and iduronic acid. They are both appreciably destroyed by the strong mineral acids used for the hydrolysis of the mucopolysaccharides. They are identified by colorimetric methods, by oxidation to the corresponding saccharic acids, or by reduction to the hexoses.

Another approach to the analysis of the mucopolysaccharides is their susceptibility to enzymes, e.g. hyaluronidases.

Information on the structure of the mucopolysaccharides has been obtained by methylation, by a study of their infrared spectra and by the isolation of split products after acid or enzymic hydrolysis.

Hyaluronic Acid and Hyaluronic Acid–Protein Complexes. By H. J. ROGERS. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

A high-molecular-weight substance which gave viscous solutions was first isolated from vitreous humour of eyes from cattle by Meyer & Palmer (1934). This substance yielded glucosamine and acetic acid on acid hydrolysis; tests for hexuronic acid in the unhydrolysed material were positive. Subsequent work showed that these three components were present in approximately equimolar amounts. Smaller amounts of sulphur were also present in most of the early preparations; the substance itself, however, was regarded as sulphur-free. The name hyaluronic acid was given to this acidic, glucosamine-containing sulphur-free compound.

Subsequently hyaluronic acid has been isolated from a wide range of tissues of mesodermal origin, and from some bacterial cultures. Its probable presence and distribution in tissues has also been examined by cytochemical techniques. It can now be specified as a high-molecular-weight substance consisting of equimolar amounts of *N*-acetylglucosamine and glucuronic acid and, apart from chitin, is the hexosamine-containing polymer about which most is known.

One of the two repeating glycosidic bonds present in hyaluronic acid is contained in 3-*O*-β-glucuronosyl-*N*-acetylglucosamine; a disaccharide of glucuronic acid and *N*-acetylglucosamine linked by this bond has been isolated from hyaluronic acid by partial acid hydrolysis and its structure unambiguously established. The bond between the *N*-acetylglucosamine and the hexuronic acid is less well established. At the moment the evidence seems to favour a β-1:4-linkage, despite the absence of action of periodate on the polymer, which had previously been interpreted as pointing to the absence of vicinal hydroxy groups and thus exclusion of the 1:4-linkage.

The earlier methods of isolation led to material which gave solutions of relatively low viscosity. Methods have, however, been evolved which produce material giving solutions of very much higher viscosity and which demonstrate great shear-dependence of viscosity when examined in the Couette co-axial type of viscometer. Some of these preparations contain considerable amounts of protein and the role of this protein in maintaining the structure of the mucopolysaccharide will be discussed.

REFERENCE

Meyer, K. & Palmer, J. W. (1934). *J. biol. Chem.* **107**, 629.

Uridine Diphosphate Sugars and the Synthesis of Glycosidic Bonds. By I. D. E. Storey.
(*Department of Chemistry, The University, King's Buildings, Edinburgh* 9)

The discovery of uridine diphosphate glucose by Leloir and his co-workers in 1949 was followed by that of a number of related nucleotides, which differed mainly in the nature of the sugar residue. This has resulted in the opening up of an entirely new chapter in carbohydrate metabolism, particularly in regard to glycoside synthesis. In recent years not only have the origin and interconversion of many sugars been considerably clarified, but also a beginning has been made in understanding the biosynthesis of certain heteropolysaccharides. This review will be devoted to considering the nature of the nucleotides, their biosynthesis, their part in the transformation of various sugars and their part in the formation of glycosidic bonds.

The nucleotides so far described are generally derivatives of uridine, linked through a pyrophosphate group to the reducing group of the sugar, which may be a hexose, pentose, uronic acid, acetylamino sugar or other component. Two compounds derived from guanosine have been described, whilst others containing cytidine and thymidine have been isolated from micro-organisms. It is to be expected that more such compounds will be isolated, and the possibility exists that some may be derived from bases other than the above.

The biosynthesis of uridine diphosphate glucose from α-glucose 1-phosphate and uridine triphosphate is catalysed by a pyrophosphorylase. Uridine diphosphate *N*-acetylglucosamine is similarly formed. From both these nucleotides, by inversion at C-4 of the sugar, the corresponding galactose derivatives are obtained. Oxidation of uridine diphosphate glucose gives uridine diphosphate glucuronic acid, which in micro-organisms may be converted into the galacturonic acid derivative, and in plants decarboxylated to pentoses. Some progress has also been made in elucidating the synthesis of nucleotides containing muramic acid.

Glycosyl transfer from uridine nucleotides was first shown for glucuronide synthesis, and subsequently for that of a number of disaccharides. Of particular interest is the formation in some of these reactions of β-glycosidic bonds, since the uridine diphosphate sugars are thought to have the α-configuration. When, as now seems likely, the glycosyl groups of these compounds are incorporated into mucopolysaccharides, a similar inversion must take place.

Some Aspects of the Biochemistry of Sulphated Mucosubstances. By P. W. KENT. (*Department of Biochemistry, University of Oxford*)

As in the biosynthesis of phenolic sulphates, the sulphate group of esterified mucosubstances is derived from SO_4^{2-}, which, *in vivo*, may be available as such or can be formed metabolically from sulphur-containing amino acids. In both types of ester, a common enzymic pathway, involving two steps, leads to the formation of 'active sulphate':

adenosine triphosphate (ATP) + SO_4^{2-} → adenosine 5′-sulphatophosphate (APS) + pyrophosphate

APS + ATP → adenosine 3′-phosphate 5′-sulphatophosphate (PAPS) + adenosine diphosphate.

'Active sulphate' (PAPS), the structure of which has been confirmed by chemical synthesis, has been isolated from a number of mammalian tissues. Its formation is known also in fungi.

Enzymic transfer of 'sulphate' (or more probably $-SO_3H$) from PAPS has been found with a variety of acceptors. In particular, phenols (excluding tyrosine, however), steroids, choline, bilirubin, β-naphthylamine and aniline, and recently several simple monohydric alcohols, are found to be sulphated in this way. Transfer to monosaccharides or their uridine diphosphate derivatives has, however, not been observed nor have any low-molecular-weight, sulphated intermediates (other than PAPS) been found to participate in systems synthesizing sulphated polymers. Evidence suggests that in these systems esterification of pre-formed oligo- or poly-saccharides occurs. It is still not clear whether transfer from PAPS to carbohydrate takes place directly or whether further intermediate steps are involved as suggested by the model system in which a glucose sulphate is synthesized via a phenolic sulphate by enzymes of *Charonia lampas*.

Biosynthesis of ^{35}S-labelled chondroitin sulphate by cell-free preparations have been reported with cell-free preparations of embryonic chick cartilage and beef embryonic cartilage; PAPS functioned as the sulphate donor and uridine diphosphates of glucuronic acid and *N*-acetylamino sugars participated. The pattern of polymerization therefore appears to resemble that reported for hyaluronic acid, chitin and cellulose, where uridine diphosphate sugars function as glycosyl donors.

The presence of uridine diphosphate sugars and the formation of PAPS has been detected also in mucous cells of the intestinal tract. *N*-Acetylneuraminic acid occurs extensively in the mucosubstances formed by these tissues. Recent evidence indicates that this acid is related biologically as well as chemically to D-mannosamine, which can be formed from uridine diphosphate *N*-acetylglucosamine. Rat-liver enzymes catalyse a reaction between pyruvate and *N*-acetyl-D-mannosamine, giving *N*-acetylneuraminic acid.

Three types of chondroitin sulphate (*A*, *B* and *C*), differing in chemical constitution, physical properties and susceptibility to enzymic degradation occur, with keratosulphate, in connective tissues.

In *A*, found in cartilage, nasal septa, aorta, etc., the sulphate ester is located at C-4 of the *N*-acetylgalactosamine units, whereas in *C*, found in skin, bone and tendon, the sulphate ester is at C-6. Type *B*, of which little is known biochemically, differs from the foregoing types in having iduronic acid as its uronic acid moiety. There is growing evidence of the linkage of chondroitin sulphates to non-collagenous proteins in connective tissue.

The Enzymic Degradation of Mucopolysaccharides. By P. G. WALKER. (*Department of Biochemistry, The Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex*)

The mucopolysaccharides of mammalian connective tissues which have so far been isolated and characterized comprise hyaluronic acid, chrondroitin, chrondroitin sulphates *A*, *B* and *C*, heparin, heparitin sulphate and keratosulphate. The structure of hyaluronic acid and the chrondroitins is largely known. They contain β-glucuronosyl or β-iduronosyl and β-glucosaminyl or β-galactosaminyl linkages; the hexosamine nitrogen is acetylated and only ester sulphate groups are present. Heparin probably contains α-glucuronosyl and α-glucosaminyl linkages and has both ester sulphate and sulphamido groups. In heparitin sulphate the glycosidic linkages are probably the same as those in heparin; sulphamido acetamido and ester sulphate groups are present. The structure of keratosulphate is unknown.

Enzyme preparations from a variety of animal or microbial sources have been described which, *in vitro*, hydrolyse these linkages, either in the polysaccharides themselves, or in oligosaccharide or simple synthetic substrates. The review is concerned with the properties and specificity of these enzymes, with special reference to their employment as reagents in structural studies.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **392nd Meeting** *of the Society was held in Lecture Theatre D in the School of Chemistry, University of Leeds, on Friday,* 25 *March* 1960, *when the following papers were read:*

COMMUNICATIONS

The Rate of Incorporation of ^{32}P into Milk and Casein by the Perfused Udder. By D. C. Hardwick, J. L. Linzell and Susan M. Price. (*Institute of Animal Physiology, Babraham, Cambridge*)

Although isotopes have been widely used to study milk synthesis by the perfused udder, the low rate of incorporation into milk that may be inferred from the high activities of the substrates used is difficult to reconcile with the report of Tindal (1957) that the rate of milk secretion is 34–79% of normal in the first hour. Moreover, by using ^{32}P, Barry (1952) has calculated that in one 6·5 hr. perfusion only 3% of the milk and 2% of the casein obtained were actually synthesized during the perfusion.

Goat udders perfused entirely without substrates, using goat red cells in goat albumin–Krebs buffer, (Cargill-Thompson, Drury, Hardwick, Linzell & Tucker, 1958) can produce 28–68% of normal yield in the first 2 hr. and at lower rates for a further 2–4 hr.; they produce much more milk between 2 and 19 hr. if given substrates.

After adding 50 μc of $^{32}PO_4^{3-}$ to the initial perfusate in 8 experiments using glucose, acetate, amino acids, calcium β-hydroxybutyrate and phosphate as substrates, the specific activity of the plasma phosphorus fell steadily for 3–5 hr. and thereafter more slowly. Activity appeared in the inorganic phosphate of the milk at the first hour and rose to a maximum at 3–5 hr. It did not appear in the casein phosphorus until 2–3 hr., and then rose roughly parallel to the specific activity of the inorganic phosphate of the milk, reaching 50–100% of this at 5–8 hr. Although the specific activity of the milk inorganic phosphate did not always reach the levels in the plasma or lymph, it was generally equal to the final level in the tissue. Similar results were obtained when $^{32}PO_4^{3-}$ was infused continuously in five experiments.

Thus milk obtained in the first 2 hr. of perfusion is largely formed from precursors already in the gland. This is to be expected since the period between the uptake of $^{32}PO_4^{3-}$ and the appearance of labelled casein is 1–2 hr. in the whole animal (Aten & Hevesy, 1938; Barry, 1952). However, the results suggest that in spite of the low total yield of milk (average about 20% of normal in 10 hr.), milk casein can be largely synthesized from the precursors provided. The slight transfer of ^{32}P to preformed casein by mammary particles (Schmidt & Davidson, 1956; Sundarurajan, Sampath-Kumar & Sarna, 1957) seems unlikely to account for our findings.

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A New Lipolytic Enzyme in Pancreatic Extracts. By D. A. Hall. (*Department of Medicine, University of Leeds*)

During the purification of elastase (Hall & Czerkawski, 1959) relatively large quantities of a second fraction (AES) containing the enzyme E_1 (Hall, 1957) or elastomucase (Saxl, 1957) were obtained in a partially purified state. This fraction has been employed as a component of lipid-clearing systems *in vitro*, both in tissue (Saxl, 1957) and in plasma (Hall, 1958), and more recently *in vivo* (unpublished results), thus indicating that the active component may possibly be lipolytic in nature. Such activity has now been measured directly by an adaptation of the method of Katz (1957) using Tween 20 as substrate. A well-characterized lipase fraction from pancreas was examined simultaneously.

The reaction due to AES only commences after a marked period of lag, the duration of which is dependent on the age and concentration of the

substrate, the concentration of the enzyme and the ionic strength and nature of the buffer. Therefore the usual parameters of velocity and substrate concentration have to be replaced by maximum values obtained from the sigmoid-shaped reaction-time curve. Both enzymes show evidence of inhibition by substrate and this is borne out by the relation between $1/v$ and $1/[S]$. The minima in curves relating these two functions are independent of enzyme concentration but occur at different values of $[S]$ for each enzyme, thus indicating a significant dissimilarity between the two. Differences can also be demonstrated by a study of the reactions of the two enzymes in the presence of inhibitors. The response of the lipase and AES to eserine, Cetavlon, heparin and serum protein is to be reported. From these results, and especially from the effect of serum protein on the activity of AES, it may be deduced that the lipolytic activity of this material is quite dissimilar to that of the pancreatic lipase and resembles that of post-heparin lipoprotein lipase.

The major active component of AES acts synergistically with elastase and also facilitates the liberation of polysaccharide from whole elastic tissue. The apparent antithesis between the lipolytic activity referred to above and this mucolytic activity can be resolved if one assumes that the polysaccharide in elastic tissue is either bound to the protein of the elastic fibre through lipid or by direct ester linkages which are also susceptible to attack by a lipolytic enzyme.

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The Reaction Between Elastin and Elastase. By D. A. Hall and J. W. Czerkawski. (*Department of Medicine, University of Leeds*)

Following preliminary observations, which indicated that the adsorption of various substances on to the surface of elastin appreciably altered the course of the reaction between this substance and elastase, powdered elastin from ox ligament was subjected to the action of various chemical reagents in order to modify the small number of polar side chains which are present. The processes of esterification and acetylation were used to mask the carboxyl, amino and hydroxyl groups and the susceptibility of the modified substrate to elastolysis was measured both before and after mild saponification. The above reactions were also supported by others such as deamination, introduction of new carboxyl groups and the effect of calcium ions, and the effect of such side-chain modification was extended to include considerations of the adsorption of elastase on to its substrate.

It could be concluded that side-chain carboxyl groups are essential both for the adsorption of elastase and for the subsequent reaction. Aliphatic hydroxyl groups are also necessary for the enzymic reaction, but the phenolic hydroxyl of tyrosine appears to be unimportant. Blocking or replacement of amino groups results in an increased enzymic activity and this can be explained by a mechanism involving the two reactions postulated by Hall & Czerkawski (1959), one resulting in the dissolution of the protein and the second in its subsequent degradation.

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Bacterial Oxidation of Phenylalanine and Phenylacetic Acid. By P. J. Chapman and S. Dagley. (*Department of Biochemistry, University of Leeds*)

The results of experiments with non-proliferating suspensions have suggested that phenylalanine is oxidized by a vibrio by conversion successively into phenylpyruvic, phenylacetic and homogentisic acids (Dagley, Fewster & Happold, 1953). Cell-free extracts that catalyse reactions of these compounds have now been obtained from vibrios grown with phenylalanine as sole source of carbon and then disrupted in the Hughes press. Thus, glutamate was formed when extracts were incubated with phenylalanine, α-oxoglutarate and pyridoxal phosphate; also, phenylpyruvate was oxidized by extracts with the uptake of 0·5 mol.prop. of oxygen, presumably to phenylacetic acid, although this has not yet been firmly identified. Dialysed extracts required diphosphopyridine nucleotide for activity

and phenylacetaldehyde appears to be an intermediate; thus, phenylacetaldehyde was also oxidized (0·5 mol.prop. of oxygen) and phenylpyruvic acid was decarboxylated in an atmosphere of N_2 (about 1 mol.prop. of carbon dioxide). Although the vibrio grows well with phenylacetic acid as carbon source, phenylalanine is not so readily utilized and results will also be reported for strains of *Pseudomonas* which serve as a more convenient source of cell-free extracts.

Our results do not support the view that *o*-, *m*-, or *p*-hydroxyphenylacetic acids are intermediates in oxidation of phenylacetate by the vibrio, but extracts from cells grown with phenylacetate contain homogentisicase. Extracts which oxidized homogentisic acid have been obtained from species of *Pseudomonas* grown with tyrosine (Suda & Takeda, 1950) or phenylacetic acid (Kunita, 1955), but a full study of the course of the reaction has apparently not been reported for bacterial extracts. After dialysis for 6 hr., extracts from the vibrio oxidized homogentisic acid (1 mol.prop. of oxygen) on addition of Fe^{2+} ions and spectroscopic examination showed the presence of maleyl acetoacetate which Knox & Edwards (1955) have shown to be a product of oxidation of homogentisic acid by mammalian preparations. The yield of maleyl acetoacetate was increased for longer periods of dialysis. Cells adapted to utilize *p*-hydroxyphenylacetic acid were also capable of oxidizing homogentisic acid readily.

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An Enzymic Method of Estimating L-Tryptophan and the Interference by a 3-Indolinylidene-3-indolyl-4-pyridylmethane. By T. A. Scott (introduced by F. C. Happold). (*Department of Biochemistry, University of Leeds*).

A rapid and almost quantitative conversion of L-tryptophan into indole, pyruvic acid and ammonia can be effected, under optimum conditions, by tryptophanase. Suitably grown cells of *Escherichia coli* contain a very active tryptophanase, which is obtained in a cell-free state after acetone drying of the cells and extraction with water (Happold, 1950).

For the detection of indole in bacterial cultures, Happold & Hoyle (1934) advised extraction with light petroleum (b.p. 40–60°) prior to testing with Ehrlich's *p*-dimethylaminobenzaldehyde reagent. By optical measurement of the rosindole colours, the test was made quantitative.

A procedure for the estimation of L-tryptophan has been designed, in which the tryptophan is converted into indole by tryptophanase in the presence of its coenzyme pyridoxal 5-phosphate. The indole is determined by a modification of the original method of Happold & Hoyle (1934). The volumes of aqueous and organic phases are standardized during extraction to obviate the error due to partition. For low concentrations of indole the Ehrlich reagent is replaced by its cinnamic analogue, *p*-dimethylaminocinnamic aldehyde. The intensity of the green colour ($\lambda_{max.}$, 625 mμ) given by indole with this reagent is $2\frac{1}{2}$ times that of the red colour ($\lambda_{max.}$, 570 mμ) formed with the Ehrlich reagent (Turner, 1959).

Protein is removed from incubation mixtures before extraction of the indole. Trichloroacetic acid serves as the protein precipitant and under the acid conditions thus created indole reacts with pyridoxal phosphate, by condensation and an atmospheric auto-oxidation, to give a red pigment. Investigations with model pyridine compounds have shown the pigment to be a 3-indolinylidene-3-indolyl-4-pyridylmethane. Its formation is inhibited, and the subsequent loss of indole prevented, by the addition of dimedone, which combines with the aldehyde group of pyridoxal phosphate and renders it unavailable for reaction with indole.

An accuracy of $\pm 2\%$ is claimed for the range 2·0–60·0 μg. of L-tryptophan. The method has been successfully applied to the measurement of free L-tryptophan in growing bacterial cultures, in the amino acid pool of *E. coli* and in the tissues of the larvae of *Calliphora erythrocephala*.

The author is indebted to the D.S.I.R. for a grant and to Professor F. C. Happold, Professor A. Butenandt, and Dr E. Biekert for their help and encouragement.

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The Ribonuclease Activity of *Micrococcus lysodeikticus*. By G. R. Barker and M. Cannon. (*Department of Chemistry, The University, Manchester* 13)

As a preliminary to attempting to establish the function of ribonucleases in metabolic processes, we have determined the ribonuclease activity in *Micrococcus lysodeikticus* at different stages of growth. Cells were lysed with lysozyme, and, after centrifugation, ribonuclease activity was determined at pH 7·2 by the method of Dickman, Aroskar & Kropf (1956). Very low activity was found in cultures in the lag phase, but during exponential growth, activity increased proportionately with total cell count, indicating an approximately constant activity per cell in this phase of growth. At the onset of the stationary phase, ribonuclease activity continued to rise linearly, indicating an increase in activity per cell. This was maintained for a short period only, the activity remaining constant again when the cell population became constant, or nearly so.

It seems likely that *de novo* synthesis of ribonucleic acid involves polymerization of nucleoside di- or tri-phosphates. On the other hand, it has been suggested (Brody, 1957; Reddi, 1959) in connexion with both mammalian tissue and virus-infected leaves that ribonucleases may be concerned with synthesis as well as breakdown of ribonucleic acid.

Our results suggest that high ribonuclease activity is associated with a phase of growth of *M. lysodeikticus* when metabolic turnover begins to predominate over net synthesis. After the completion of our experiments, a similar study was reported by Nishimura & Nomura (1959), who obtained similar results with *Bacillus subtilis*. These workers, however, consider the ribonuclease to be an extracellular enzyme. We have reinvestigated the ribonuclease of *M. lysodeikticus* and find no extracellular enzyme of this type. It appears that ribonuclease is able to penetrate certain bacterial cells (Groth, 1956) and it is therefore possible that the enzyme was present in the medium of *B. subtilis* due to leakage from the cells. However, under certain conditions, lysis of *B. subtilis* may take place during growth (Richmond, 1959) and it is suggested that ribonuclease is properly to be regarded as an intracellular enzyme. Our results are in agreement with the hypothesis that the enzyme is concerned with processes of breakdown and re-synthesis associated with metabolic turnover of ribonucleic acid.

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Acetylcholinesterase in Micro-organisms Possessing Simple Flagella or Cilia. By J. Tibbs. (*Department of Biomolecular Structure, University of Leeds*)

It is possible that ciliary and flagellar movements are controlled by a mechanism involving the release and breakdown of acetylcholine. Acetylcholinesterase has been reported as being present in the gill plates of *Mytilus edulis* (Bülbring, Burn & Shelley, 1953), in the pellicle of *Tetrahymena pyriformis S.* (Seaman, 1951), in pig spermatozoa (Sekine, 1951), and Mann (1954) mentions work identifying a specific acetylcholinesterase in the tails of ram sperm. Seaman (1955) considers that the enzyme in *Tetrahymena* is probably associated with a subpellicular arrangement of neurofibrils. However, cilia adhering to the pellicular fragments could have given rise to this activity and the presence of such activity in ram-sperm tails may be held as giving support to this view. On the other hand, whilst an acetylcholine mechanism might be located exclusively in the cell body of systems carrying simple cilia with only the basic (9+2) pattern of fibrils, such a mechanism could well extend down, or be located in, the more complex tails of mammalian sperm.

The present communication is concerned with acetylcholinesterase activity and its localization in systems possessing simple cilia.

No activity could be detected in homogenates of *Polytoma uvella*, *Polytomella caeca* and in *Tetrahymena pyriformis W*. This last result conflicts with the result of Seaman & Houlihan (1951).

Spermatozoa from brown trout (*Salmo trutta*) and perch (*Perca fluviatilis*), in common with other fish sperm, possess tails which have only the basic (9+2) system of fibrils. These spermatozoa split

acetylcholine to an extent varying from 0·02 to 0·14 μg./hr./mg. at 37°. In perch sperm this activity is located in the head, the tail fraction being inactive. The failure to detect activity in the first organisms mentioned probably reflects a low level of activity in these preparations, whilst the results from fish sperm suggest that the basic (9+2) system plays no part in acetylcholine breakdown by ciliated systems and that any electrical control of movement in organisms possessing only simple cilia or flagella is exerted by the head.

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Purification of Citrase and the Enzymic Determination of Citrate. By T. J. Bowen and C. Siva Raman. (*Department of Biochemistry, University of Leeds*)

Ultracentrifugal analysis has shown that several ribonucleoprotein components are common to cell-free extracts from different bacterial species (Schachman, Pardee & Stanier, 1952). Dagley & Sykes (1958) produced evidence against the view of several authors that the '40s component' carried various enzymes (see, for example, Tissières, 1954) but they observed that citrase, glutamate decarboxylase and β-galactosidase sedimented in crude extracts at the same rate as a boundary (12s) in schlieren diagrams. They could not decide whether this was due to the attachment of the enzymes to the same ribonucleoprotein particle, or whether the molecules of the enzymes were large enough and present in sufficient amounts to account for their observations. For this and other reasons we have improved upon the methods of Dagley & Dawes (1955) for purification of citrase from *Escherichia coli* by a sequence of operations involving removal of nucleic acids by addition of manganous sulphate, heating to 50°, treatment with calcium phosphate and alumina gels, precipitation with ammonium sulphate and chromatography on columns of diethylaminoethylcellulose. A preparation was obtained free from nucleic acids and almost homogeneous in the ultracentrifuge. The enzyme sedimented at 16s. There is again a similarity with β-galactosidase, purified by Cohn (1957). Evidently in both cases *E. coli* may synthesize large enzyme molecules in relatively large amounts in order to catalyse the breakdown of single substrates.

We have not purified the citrase from *Aerobacter aerogenes* so extensively, but treatment of crude extracts to remove enzymes catalysing the oxidation of reduced diphosphopyridine nucleotide (DPNH) gave a preparation suitable for determination of citrate at concentrations lower than those to which the method of Dagley & Dawes (1953) is applicable. Citrate (10–80 μg.) is incubated in a silica cell with 0·2 ml. of extract in a total volume of 3 ml. at pH 7·4. It is converted into pyruvate by the citrase and oxaloacetate decarboxylase contained in the extract. DPNH is added, followed by lactate dehydrogenase, which causes a fall in extinction at 340 mμ. For each mole of DPNH oxidized, 1 mole of citrate disappears; 0·1 μmole of citrate corresponds to a fall in extinction of 0·207 (cell path, 1 cm.). Glutamic and α-oxoglutaric acids inhibited formation of pyruvate but a wide range of common biochemical reagents including *iso*citrate did not interfere; *cis*-aconitate reacted slightly.

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Changes in Microsomal Enzyme Systems in the Liver of Newborn Rabbits. By MARJORIE FLINT, G. H. LATHE, T. R. RICKETTS and GERTRUDE SILMAN. (*Department of Chemical Pathology, University of Leeds*)

The activity of the bilirubin transglucuronosylase system is low in newborn rabbits, as previously shown in premature humans (Lathe & Walker, 1958). It reaches adult levels in 1–2 weeks.

Brown & Zuelzer (1958) showed that in guinea-pig foetuses the concentrations of the cofactor, uridinediphosphoglucuronic acid, was also low. However, in the newborn rabbit the rate of conjugation of *o*-aminophenol demonstrated that adequate uridinediphosphoglucuronic acid was available. We concluded that the limiting factor for conjugation of bilirubin was the transferase.

Transglucuronosylase is located in liver microsomes (Schmid, Hammaker & Axelrod, 1957). The deficit in the newborn period may, therefore, be due either to a smaller amount of microsomes or to a difference in their structure. If the former explanation were true, all microsomal enzymes would show a similar pattern of development. A wide variety of patterns was found among the following enzymes: *o*-aminophenol transglucuronosylase, glucose 6-phosphatase, esterase, acetylcholinesterase, cholinesterase, diphosphopyridine and triphosphopyridine nucleotide–cytochrome *c* reductases and diphosphopyridine and triphosphopyridine nucleotide diaphorases. In liver slices the *o*-aminophenol transglucuronosylase showed a development curve similar to that for the bilirubin-conjugating enzyme, but took longer to reach the adult level. This may reflect enzyme or cofactor production. At birth glucose 6-phosphatase was twice the adult value and fell within 4 weeks. Esterase (substrate, ethyl butyrate) remained very low for 2 weeks, then rose to reach the adult level at 6 weeks. The capacity to hydrolyse acetyl-β-methylcholine was constant at the adult level, while that for benzoylcholine rose gradually over the first 4 weeks. Cytochrome *c* reductases and diaphorases started at adult levels, but showed an upward excursion at 2–3 weeks, followed by a return to former levels.

Preliminary attempts were made to separate microsomes by differential centrifugation. In the young a larger proportion of microsomal material was not sedimentable at 15 000 ***g***, but came down at 103 000 ***g***. This fraction contained a disproportionately small amount of glucose 6-phosphatase. As Hultin (1957) has shown that this enzyme is in the membranous fraction of the reticulum, it may be that young liver contains a larger proportion of unattached granular elements than does adult liver. This was suggested by the electron-microscope studies of Palade (1955).

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Observations on the Absorption Spectra of Polysaccharide Iodine Complexes. By A. R. ARCHIBALD, D. J. MANNERS and A. WRIGHT. (*Department of Chemistry, University of Edinburgh*)

In the course of structural studies on starch-type polysaccharides, we have measured the absorption spectra of the iodine complexes of more than 50 different samples of amylopectin and glycogen. Using conditions similar to those of Peat, Whelan, Hobson & Thomas (1954), in which the absorption spectra of 0·01 % polysaccharide solution stained with a solution of 0·02 % iodine in 0·2 % potassium iodide is measured in a Unicam SP. 500 spectrophotometer, values for the wavelength of maximum absorption ($\lambda_{max.}$) and the extinction (absorption value) at this wavelength ($E_{max.}$) have been obtained.

Glycogens from various biological sources gave $\lambda_{max.}$ values in the range 420–490 mμ with $E_{max.}$ 0·1–0·4. The iodine-staining power increased generally in the order glycogen β-limit dextrin, invertebrate glycogen, mammalian-liver glycogen and mammalian-muscle glycogen, but there was no correlation between $\lambda_{max.}$ or $E_{max.}$ values and the degree of branching, or the relative lengths of the exterior and interior chains.

With amylopectins, the corresponding $\lambda_{max.}$ and $E_{max.}$ values were 530–550 mμ and 0·8–1·2, respectively, and there was no apparent relation between iodine-staining power and branching characteristics. Floridean starches gave absorption spectra with $\lambda_{max.}$ about 500 mμ and $E_{max.}$ about 0·7.

Amylopectin and glycogen differ markedly in iodine-staining properties in two additional respects. First, during the β-amylolysis of glycogen, a decrease in both $\lambda_{max.}$ and $E_{max.}$ is observed, whereas for amylopectin the $\lambda_{max.}$ of the intermediate and limit dextrins is unaltered. The type of

iodine binding in the two polysaccharides must therefore differ, and in the case of amylopectin it is suggested that the length of the interior chains is a controlling factor. Secondly, the addition of ammonium sulphate (cf. Schlamowitz, 1951) to the polysaccharide–iodine–iodide solution caused an increase in iodine-staining power. With amylopectins, the $\lambda_{max.}$ is unaltered but $E_{max.}$ is increased by 10–30%; with glycogens, $\lambda_{max.}$ is increased by about 40 mμ, and $E_{max.}$ is increased to the range 0·8–1·1. In these solutions, the dehydrating action of ammonium sulphate may facilitate the binding of iodine by a proportion of the exterior chains of glycogen.

We wish to thank Professor E. L. Hirst, F.R.S., for his interest in this work.

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The Metabolism of Glyoxylate by Cell-free Extracts of *Pseudomonas fluorescens*. By R. P. Hullin and H. Hassall. (*Department of Biochemistry, University of Leeds*)

The operation of the glyoxylate cycle in a wide range of micro-organisms has recently been reviewed (Kornberg, 1959). The net effect of each turn of this cycle is the formation of one molecule of C_4-dicarboxylic acid from two molecules of acetate, the key enzymes being *iso*citratase and malate synthetase. *iso*Citratase is present in high concentration only in organisms grown on acetate, or direct precursors of acetate, as sole carbon source.

A further metabolic cycle has been described whereby micro-organisms utilizing butane-2:3-diol or acetylmethylcarbinol as carbon source degrade the substrate to acetate (Juni & Heym, 1956). It was therefore of interest to see whether the two cycles are coupled together in bacteria grown on butane-2:3-diol, as has been suggested, but not established experimentally (Dagley, 1959). If the two cycles are linked, it is to be expected that *iso*citratase should be present in high concentration in cells grown on butane-2:3-diol as carbon source.

Cell-free extracts of a strain of *Pseudomonas fluorescens* grown on butane-2:3-diol possessed an *iso*citratase activity as high as when grown on acetate and about 20 times as high as when the cells were grown on succinate. Such extracts also exhibited malate synthetase activity; this enzyme does not appear to be developed adaptively since extracts of cells of the same strain grown on succinate also showed this activity. Malate accumulation was demonstrated by incubation of dialysed extract with glyoxylate, acetate, adenosine triphosphate and magnesium ions in an atmosphere of nitrogen.

Crude extracts of the organism grown on butane-2:3-diol, acetate or succinate convert glyoxylate to glycollate and carbon dioxide without further additions and without uptake of oxygen. Whilst no direct evidence of the metabolic pathways involved in this dissimilation of glyoxylate has yet been obtained, it has been demonstrated that pyruvate accumulates in the reaction mixture when extracts are incubated aerobically with malate in the presence of semicarbazide. In the absence of semicarbazide, pyruvate is itself rapidly metabolized by crude extracts. It is suggested that the carbon dioxide may arise by a pathway similar to that demonstrated by Kornberg & Sadler (1960), which required catalytic amounts of acetyl-coenzyme A and that the reduced diphosphopyridine nucleotide formed is re-oxidized by the glyoxylate → glycollate reaction.

The dissimilation of glyoxylate to glycollate and carbon dioxide has been confirmed using [$^{14}C_2$]-glyoxylate.

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Synthesis of α-1:6-Glucosidic Linkages by a Transglycosylase from Potato. By M. ABDULLAH and W. J. WHELAN. (*Lister Institute of Preventive Medicine, London, S.W.* 1)

The enzymic conversion of α-1:4-glucosidic bonds into α-1:6-bonds is effected in the potato by Q-enzyme, which converts amylose into amylopectin and which acts rapidly on amylose chains only when their degree of polymerization is greater than 40 glucose units (see review by Whelan, 1958). A second enzyme capable of performing the same type of transformation with much simpler substrates has now been discovered in the potato. It acts on maltose as follows:

$$2 \text{ maltose} \rightarrow \text{panose} + \text{glucose} \qquad (1)$$
(panose = 6^2-α-glucosylmaltose)

This type of catalysis has previously been found in enzyme preparations from *Aspergillus* spp. (Pan, Nicholson & Kolachov, 1951; Pazur & French, 1952; Barker & Carrington, 1953), in *Penicillium chrysogenum* (Saroja, Venkataraman & Giri, 1955), *Cladophora rupestris* (Duncan & Manners, 1958) and *Tetrahymena pyriformis* (Archibald & Manners, 1959).

The following reactions are also mediated by the potato enzyme preparation:

2 panose → 6^2-α-isomaltosylmaltose + maltose, (2)

2 isomaltose → isomaltotriose + glucose, (3)

2 isomaltotriose → isomaltetraose + isomaltose (4)

All products except 6^2-α-isomaltosylmaltose and all unchanged substrates have been characterized as crystalline derivatives.

The enzyme preparation is therefore capable both of transforming α-1:4-bonds into α-1:6-bonds [reaction (1)] and of redistributing α-1:6-bonds [reactions (2), (3) and (4)]. The evidence suggests that reactions (2), (3) and (4) are reversible, while reaction (1) is essentially irreversible. Such behaviour also occurs with the enzyme preparation from *A. oryzae* (Pazur, 1954). The question arises whether both types of reaction are mediated by the same enzyme. Quantitative measurements have been made of the rates of action of the enzyme preparation on maltose and isomaltotriose, separately and in combination, both substrates being present in excess. The extent of action on each substrate in a given time is halved when they are present together, compared with the degree of conversion attained with a single substrate. From this and other evidence it is concluded that it is the same enzyme which acts on both substrates.

Until the catalytic action of the enzyme has been further defined it is proposed to refer to it as T-enzyme.

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Tetrahydropteroyltriglutamate as a Cofactor of Methionine Synthesis. By J. R. GUEST and K. M. JONES. (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The folic acid cofactor for the synthesis of methionine from homocysteine and serine by ultrasonic extracts of *Escherichia coli* PA15 can be supplied either by an extract of heated organisms (EHC) or by tetrahydropteroylglutamate (Guest, Helleiner, Cross & Woods, 1960). There are differences in the conditions under which these two sources of cofactor are active and in their properties, namely, (1) tetrahydropteroylglutamate is active only if cobalamin is added to the reaction mixture, while EHC is fully active without added cobalamin; (2) the methionine synthesis promoted by EHC is inhibited by the addition of tetrahydropteroylglutamate in the absence of cobalamin; (3) the cofactor activity of tetrahydropteroylglutamate is rapidly lost on exposure to air while that of EHC is stable under these conditions.

Wright (1960) has purified from heated extracts of *Clostridium cylindrosporum* several pteroylpolyglutamates which possess cofactor activity for the conversion of serine into glycine by enzymic extracts of *Clostridium sticklandii*. A crude mixture of these compounds is also active in methionine synthesis in *E. coli* (Szulmajster & Woods, 1960).

Tetrahydropteroyltriglutamate has been prepared by reduction of pteroyltriglutamate. Unlike the monoglutamate, it was active as a cofactor for

the synthesis of methionine by ultrasonic extracts of *E. coli* PA 15 in the absence of added cobalamin. Addition of cobalamin had no appreciable effect. The concentration of tetrahydropteroyltriglutamate required for half-maximal activity was 25 μM, compared with 150 μM for the monoglutamate in the presence of cobalamin. Tetrahydropteroylglutamate competitively inhibited the methionine synthesis promoted by tetrahydropteroyltriglutamate. Similar results were obtained with extracts of acetone-dried organisms, and also with tetrahydropteroyltriglutamate prepared from pteroyltriglutamate purified by chromatography.

N^{10}-Formyltetrahydropteroyltriglutamate, prepared by treating pteroyltriglutamate with formic acid and reducing the product, was active as a cofactor of methionine synthesis at the same concentration as tetrahydropteroyltriglutamate, but when added in larger amounts in the absence of serine showed negligible activity as a C_1-unit donor in the synthesis of methionine.

These results show that tetrahydropteroyltriglutamate and its N^{10}-formyl derivative resemble EHC rather than tetrahydropteroylglutamate in their effect upon methionine synthesis. They are, however, like tetrahydropteroylglutamate, unstable in air and so it seems unlikely that either of these compounds is, in fact, the active component of EHC.

Pteroyltriglutamate (teropterin) was kindly supplied by Dr E. L. R. Stokstad.

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The Effect of Glutathione Disulphide on the Thiol Content of Bovine-serum Albumin. By T. E. Isles and P. C. Jocelyn. (*Department of Clinical Chemistry, University of Edinburgh*)

Klotz, Ayers, Ho, Horowitz & Heiney (1958) found that a diphenyl disulphide reacted quantitatively with the thiol group of bovine serum albumin (BSA) to give a mixed disulphide, and a similar reaction involving cystamine has been described by Eldjarn & Pihl (1956). Since glutathione disulphide (GSSG) occurs in small quantities in human serum (McIlwain, 1959), the question has been investigated whether or not it also reacts with BSA. If BSA, or BSA treated with iodine to abolish the thiol group (Hughes & Straessle, 1950), is treated with *p*-chlormercuribenzoic acid (PCMB), the proteins precipitated and the remaining PCMB estimated spectrophotometrically, the results show that the amount of PCMB reacting with the protein thiol group (Boyer, 1954), but only this amount, is quantitatively removed from the solution along with the precipitated protein. Therefore, from measurements of the PCMB remaining in the supernatant, the thiol content of BSA can be estimated. The value obtained (0·65–0·67 –SH group per molecule of BSA) is in good agreement with that found by other workers (e.g. Carter, 1959). Furthermore, since the reaction of glutathione (GSH) with PCMB involves a large increase in absorption at 255 mμ, any increase in absorption obtained by adding PCMB after precipitating BSA from BSA–GSH mixtures measures the concentration of GSH in the supernatant. In addition, GSH and GSSG (the latter after enzymic reduction with a glutathione reductase preparation) have also been measured by a modification of the alloxan method (Patterson & Lazarow, 1955).

When BSA (1 mole) and GSSG (up to 20 moles) are incubated at pH 7·4 for 15 hr. under nitrogen, there is no significant decrease found in the protein thiol titre when allowance has been made for the absorption due to GSSG. Also, no significant formation of GSH can be detected using both the methods described, and GSSG is recovered after incubation to the same extent as in control solutions incubated without the addition of BSA. The addition of cupric ions has no effect on the reaction.

This finding that GSSG does not react with the thiol group of BSA suggests that the use of inhibition of the activity of enzymes by GSSG as a criterion of thiol-group dependence may not be fully justifiable.

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Isolation of Keratosulphate from Bovine Nucleus Pulposus and Cornea. By P. F. Lloyd, G. P. Roberts and K. O. Lloyd. (*Department of Chemistry, University College of North Wales, Bangor, Caernarvonshire*)

Pure keratosulphate was required for enzymic and structural studies. This mucopolysaccharide, in crude form, was isolated earlier (Hall, Lloyd, Happey, Horton & Naylor, 1957) by aqueous extraction of human nucleus pulposus, taken from a subject aged 70. Application of this method to young bovine nucleus pulposus has now given different results, extraction with water furnishing mainly a mucopolysaccharide–protein complex of low electrophoretic mobility. Substantially pure keratosulphate (D-galactosamine $< 5\%$ of total hexosamine) was obtained by the following sequence: (1) aqueous extraction, (2) Sevag deproteinization, (3) digestion with trypsin, (4) digestion with hyaluronidase (cf. Kaplan & Meyer, 1959), (5) ethanol fractionation.

Fractionation with cetylpyridinium chloride (Rodén, 1956) and electrophoretic separation were attempted after stage (3). The former method gave some separation, but the latter was only effective after disruption of chondroitin sulphate–protein complex (for example, by alkali), which had survived the trypsin digestion, since the conjugated protein migrated at a similar rate to that of keratosulphate (paper electrophoresis).

Subsequently a less pure keratosulphate was obtained by essentially the method of Meyer, Linker, Davidson & Weissmann (1953). An improved column method for the fractionation of nucleus pulposus mucopolysaccharides by ethanol has been reported (Gardell, 1957).

Bovine cornea was also examined. Very little mucopolysaccharide was extracted with water (cf. Woodin, 1954) and application of the method of Meyer *et al.* (1953) (omitting treatment with Lloyd's reagent) led to incomplete separation of the mucopolysaccharide mixture. The products, which at this stage still contained unresolved mucopolysaccharide–protein (or peptide) complexes, were fractionated on a column of diethylaminoethylcellulose (Peterson & Sober, 1956; cf. Ringertz & Reichard, 1959) by gradient elution (5 mM-phosphate buffer, pH $7{\cdot}0 \rightarrow 3{\cdot}0$; $0 \rightarrow$ M-sodium chloride). The last polysaccharide-containing fractions eluted from the column yielded keratosulphate (in a discrete band) completely free from D-galactosamine.

Interpretation of our results and those of other workers (Bernardi, Happey & Naylor, 1957; Davidson & Woodhall, 1959) is possible when it is recognized that the ratio, keratosulphate:chondroitin sulphate, in nucleus pulposus, and also in other tissues, increases with the age of the subject (Hallen, 1958), and if it is also accepted that the ratio of unbound to protein-conjugated keratosulphate varies with age and physiological state of the tissue.

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Observations on the Effect of Trypsin on Elastase. By G. N. Graham (introduced by D. A. Hall). (*Department of Biomolecular Structure, University of Leeds*)

The proteolytic nature of the activity of the elastase complex has been confirmed by a number of workers, though its physiological function is still in doubt. Theories suggesting a connexion between elastase activity and the onset of arteriosclerosis have led to the study of a natural inhibitor of elastase, present in serum, which was first reported by Baló & Banga (1949). During the course of investigations into the character and properties of this inhibitor from serum it became obvious that it bore a close resemblance to one of the trypsin inhibitors in serum (Jacobsson, 1953). It is therefore apposite to consider the relationship of trypsin to the elastin–elastase system and the following observations will be of interest in calling attention to this aspect of the elastolytic reaction.

(1) Grant & Robbins (1955) have demonstrated that an inactive zymogen pro-elastase, which may

be activated by trypsin, can be extracted from fresh hog pancreas and is present in dog pancreatic juice. The author has repeated these observations on rabbit pancreas. An N-sodium chloride extract of rabbit pancreas can be shown to have no elastolytic activity but after adding small amounts of trypsin the extract becomes active.

(2) A quite highly purified specimen of elastase can be shown to have its activity enhanced by the addition of trypsin to the reaction mixture.

(3) The inhibitory action of serum on elastase may be completely reversed by the action of trypsin in a stoicheiometric manner. The complex formed between trypsin and the trypsin inhibitor of soya bean does not so reverse the inhibition of elastase by serum, though the trypsin inhibitor of soya bean is itself an inhibitor of elastase.

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Phosphatases in Seminal Plasma of the Domestic Cock. By D. J. Bell and P. E. Lake. (*Agricultural Research Council, Poultry Research Centre, Edinburgh* 9)

During investigations in connexion with attempts to preserve, *in vitro*, fertile avian spermatozoa (Lake, Butler, McCallum & McIntyre, 1958; Lake & McIndoe, 1959) we found that cock-seminal plasma possesses high acid phosphomonoesterase activity, which is unaffected by keeping diluted samples at −20°. This is surprising since birds (Lake, 1957) are devoid of those organs which, in mammals, are believed to supply the bulk of the acid phosphatase(s) to the chemically different semen of the latter phylum.

Enzymic activity against 4-nitrophenyl phosphate is especially marked and shows two nearly overlapping peaks about pH 4·8 and 5·2. The enzyme is active against both the 1- and 2-phosphates of glycerol but to a much smaller degree than against 4-nitrophenyl phosphate. In contradistinction to mammalian seminal plasma (Lundquist, 1947; Dawson, Mann & White, 1957), the avian enzyme could not be shown to hydrolyse choline *O*-phosphate. Moreover, neither Ca^{2+}, Cu^{2+} nor L-tartrate, in concentrations which inhibit human prostatic phosphatase (Abul-Fadl & King, 1949) had any effect on the avian enzyme; it was, however, completely inactivated by F^-.

Alkaline phosphatase activity against 4-nitrophenyl phosphate was also demonstrated; this is potentiated by Mg^{2+}. Evidence of the presence of phosphodiesterase active at pH 4·8 was also obtained. The activities of both monoesterases are considerably greater in the seminal plasma than in the blood plasma of the cock.

It is tempting to suggest that, although it must originate in different somatic sources, the high acid phosphatase activity in the seminal plasmas of both mammals and birds, may indicate, in these two phyla, a common physiological purpose for the enzyme.

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Histochemical Demonstration of Phosphomonoesterase Secretion in the Genital Tract of the Domestic Cock. By P. E. Lake (introduced by D. J. Bell). (*Agricultural Research Council, Poultry Research Centre, Edinburgh* 9)

In mammals the bulk of seminal acid phosphatase activity is generally derived from the prostate; the alkaline phosphatase originates in the seminal vesicles. The relative amounts of the two enzymes vary with the development of the particular accessory gland in any species (Mann, 1954). The cock possesses neither prostate nor seminal vesicles (Lake, 1957) but its seminal plasma displays high acid phosphatase activity and contains appreciable alkaline phosphatase (Bell & Lake, 1960). In view of these observations a histochemical examination of the genital tract was made to localize, if possible, the source of the enzymes concerned. Rollinson (1954) described variable phosphatase activity in histological sections of different parts of the genital tracts of various

mammals. In the bull, acid phosphatase activity was found mostly in the epididymis, the ampulla of the vas deferens and the seminal vesicles, little or none being found in the prostate.

Previously (Lake, 1957) using Gomori's method on paraffin-embedded tissues (Pearse, 1960), no convincing evidence was obtained for any intense secretion of acid phosphatase in the cock genital tract, apart from isolated areas in the vas deferens and vasa efferentia. Pearse (1960) has indicated that in the above method much of the enzymic activity may be lost. Frozen sections of various areas of the genital tract previously fixed in 10% neutral formalin (6–16 hr. at 2°) were incubated for 20–60 min. in appropriate substrate–dye mixtures at either pH 4·9 or 9·6. The substrate was sodium α-naphthyl phosphate. For acid phosphatase the liberated phenol was coupled with the stable diazonium salt of 4-benzoylamino-2:5-dimethoxyaniline (Fast Blue RR Salt, Gurr's Ltd., London). For alkaline phosphatase the coupling agent was 4-chloroanisole-2-diazonium chloride (Fast Red RC Salt, Gurr's Ltd.). These dyes have little inhibitory activity on the enzymes (Pearse, 1960).

Intense acid phosphatase activity was found in the distal regions of the cells lining the entire length of the vas deferens and certain tubules of the epididymal region. There was also some activity in the cytoplasms of the spermatids and secondary spermatocytes which might be shed into the lumen of the seminiferous tubule. Intense alkaline phosphatase activity was found only in secretions of certain tubules in the epididymal region, in the Tunica intima of all small blood vessels and in intertubular testicular tissue.

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Chromatographic Isolation of the Ethanolamine-containing Phospholipids of Rat Brain. By C. Long and D. A. Staples. (*Biochemistry Division, Physiology Department, Institute of Basic Medical Sciences, Royal College of Surgeons of England, London, W.C. 2*)

When a rat-brain lipid extract, freed from water-soluble impurities and proteolipid protein, is applied to an alumina column equilibrated with chloroform–methanol (1:1, v/v), the neutral lipids and choline-containing phospholipids are not retained. If the water content in the solvent is later increased from zero to 7% (by vol.) by means of a gradient, the cerebroside plus sulphatide fraction is eluted (Long & Staples, 1959). A further gradual increase in the water content was found to lead to the elution of an aminophospholipid fraction and the nature of this material has now been studied.

This aminophospholipid peak was completely eluted when the water content reached 11% (by vol.), as shown by the ninhydrin reaction. Summation of the analytical values on nine fractions gave the following mean molar ratios total: P:total N:ester:aldehyde, 1·00:0·99:1·51:0·53. All the nitrogen was in the form of ethanolamine, as determined by the method of Axelrod, Reichenthal & Brodie (1953), and this accounted quantitatively for virtually the whole of ethanolamine present in the lipid applied to the column. These results indicate that the material consists essentially of approximately equal parts of phosphatidylethanolamine and ethanolamine plasmalogen. However, the ratio (aldehyde + ester):phosphorus, which had an average value of 2·05 for the first 90% of the eluted material, fell to about 1·60 for the last 10%. Furthermore, we have evidence that some decomposition occurred on the column, leading to the formation of neutral lipid. Thus it seemed likely that the principal contaminents would be neutral lipid and ethanolamine lysophospholipids.

When the above fractions were combined, evaporated to dryness, dissolved in chloroform–methanol (49:1, v/v) and applied to a silicic acid column, the neutral lipid passed through the column, unaccompanied by either nitrogen or phosphorus. Gradient elution to give a final solvent composition of chloroform–methanol of 9:1 (v/v) resulted in the removal of phosphatidylethanolamine plus ethanolamine plasmalogen, with correct analytical figures and an aldehyde:phosphorus ratio of 0·54. This fraction accounted for at least 80% of the ethanolamine in the original brain-lipid extract. Finally, the remainder of the ethanolamine-containing phospholipids was eluted with chloroform–methanol–water (5:14:1, by vol.). It had the following ratio: total P:total N:ester:aldehyde, 1·00:0·95:0·92:0·18, indicating that it was essentially a mixture of lyso compounds.

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Chromatographic Separation of Serine-containing Phospholipids of Rat Brain. By C. LONG, B. SHAPIRO and D. A. STAPLES. (*Biochemistry Division, Physiology Department, Institute of Basic Medical Sciences, Royal College of Surgeons of England, London, W.C. 2*)

After removal of the ethanolamine-containing phospholipids from the alumina column (Long & Staples, 1960), elution of the serine-containing phospholipids was attempted. In pilot experiments, the only suitable solvent mixture proved to be diethyl ether–ethanol–0·04N-KOH (10:7:5, by vol.). However, it was found impracticable from a single sample of rat-brain lipid extract to elute serially the galactose-, ethanolamine- and serine-containing lipids, owing to the extensive decomposition of the latter which occurred on alumina columns of the usual dimensions (13 g.; 18 × 1 cm.) during the course of the several days required. Good results were obtained, on the other hand, when a short column (2 g. of alumina; 2·5 × 1 cm.) was used in the following manner.

The rat-brain lipid, dissolved in chloroform–methanol–water (44·5:44·5:11, by vol.) was applied to the short column, which was then eluted with 200 ml. of the same solvent within 30 min. The amino-nitrogen level, due to ethanolamine phospholipid, fell to a low value. The alkaline solvent system was then applied to the column, whereby a second amino N-containing peak was eluted in eight 15 ml. fractions. A summation of analyses gave the following mean molar ratio: total P:total N:amino-N:ester:serine:ethanolamine, 1·00:0·85:0·74:1·67:0·72:0·01. The material is thus essentially a serine-containing phospholipid, contaminated with a small amount of non-amino N and a larger amount of nitrogen-free phospholipid.

Alumina acts as a cation-exchanger, and in the above experiment 75 ml. of the alkaline solvent passed through the column before the eluate turned pink to phenolphthalein. Up to this point, there was equivalence between total P, total N, amino-N and serine, the ester:P ratio was 1·82, and the aldehyde:P ratio about 0·10. After the breakthrough of the alkali, the phosphorus content of the eluted material was appreciably in excess. This suggests that other acidic lipids of the type of phosphatidic acid and/or diphosphoinositide are being eluted in the later stages.

The fractions from the alumina column were combined, evaporated to dryness, dissolved in chloroform–methanol (2:1, v/v), and washed with aqueous potassium chloride according to Folch, Lees & Sloane-Stanley (1957). In this way, much of the excess phosphorus was removed. Finally the washed lipid, dissolved in chloroform–methanol (49:1, v/v) was applied to a silicic acid column. After removal of a small amount of neutral lipid with this solvent, the methanol content was gradually raised to 34% (v/v). An amino N-containing peak was eluted, representing about 80% of the amount applied. The total N and amino-N values were now nearly equal and, apart from the early fractions, the N:P ratio was approximately unity.

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The Metabolism of ^{14}C-labelled Vitamin D_2 in the Rat. By E. KODICEK and D. R. ASHBY. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

Four stock rats, weighing about 140 g., were each given, by stomach tube, a single dose of 1 mg. of uniformly ^{14}C-labelled vitamin D_2 (specific activity, 0·173 μc/mg.), dissolved in 0·2 ml. of arachis oil. The fate of the labelled vitamin was followed by analysing the tissues and excreta for ^{14}C. The materials were fractionated according to Kodicek (1956*a*); the ether-extractable unsaponifiable fraction contained only the ^{14}C-labelled vitamin D, as shown by radioautographs of reversed-phase chromatograms (Kodicek & Ashby, 1954). The other fractions contained ^{14}C-labelled breakdown products.

The recovery of total ^{14}C in the tissues, examined 5, 10 and 24 hr. after dosing, was as follows (results are expressed as percentage of the dose and the figures in brackets denote the ^{14}C-labelled vitamin D): in the faeces and gut contents 82 (54·6), 83 (34·5) and 78·8 (44·8); in the liver 11·1 (7·8), 6·8 (4·9), 8·0 (2·5); in the kidneys 0·8 (0·1), 0·5 (0·2), 0·3 (0·1); in stomach and colon tissue 0·52 (0·52), 0·14 (0·14), 0·18 (0·18); in duodenum and jejunum 1·17 (0·9), 0·4 (0·3), 0·4 (0·2); in ileum 3·2 (2·2), 0·9 (0·8), 0·2 (0·1). In bones after 24 hr. only 0·1% of the dose was found as vitamin D. The total recovery of ^{14}C at 5 hr. was 98·8% (66·1% as vitamin D), at 10 hr., 91·7% (40·8% as vitamin D), at 24 hr., 88·0% (48·0% as vitamin D).

The findings in liver confirm and extend previous results with non-radioactive vitamin D_2 (Cruickshank & Kodicek, 1957), showing that, as soon as 5 hr. after dosing, a maximal accumulation occurred

of both vitamin D and breakdown products. All the vitamin was free, not esterified, confirming the findings with non-radioactive vitamin D_2 (Kodicek, 1957). No indication of the presence of a ^{14}C-labelled Keton 250 (Raoul, 1958; Gounelle, 1959), derived from the ^{14}C-labelled vitamin D, was found in the biologically active fractions. The recovery in liver was lower than in rachitic rats, suggesting a greater accumulation of ^{14}C-labelled substances in the latter, in line with the observations of Blumberg, Schönholzer & Aebi (1959). The faeces contained, after only 5 hr., the major portion of the dose, about one-third to one-half as breakdown products; the latter are considered to be excreted by the body via the bile (Kodicek, 1956*b*). The kidneys had small, but significant amounts of vitamin D, particularly at 10 and 24 hr. after dosing, but the greater part of the ^{14}C was in the form of breakdown products. The intestines showed a similar distribution of ^{14}C-labelled vitamin D, as reported with non-radioactive vitamin D_2 (Cruickshank & Kodicek, 1958), suggesting that the ileum is the site of absorption of vitamin D. The bones accumulated less vitamin D than bones of dosed rachitic rats (Kodicek, 1960). The nature of the breakdown products is to be reported.

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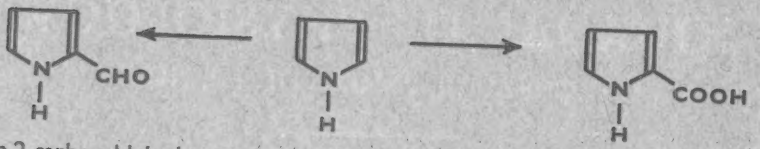

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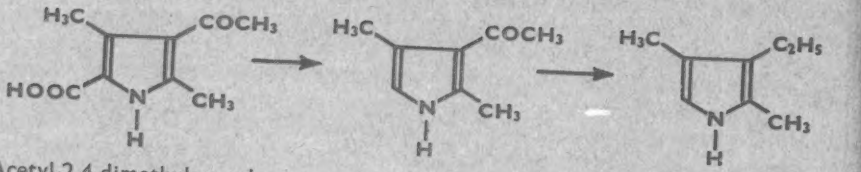

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-5-carboxylic acid

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